THE REDUCTION OF THE DISULFIDE BONDS OF RIBONUCLEASE

Lisa Amelia Steiner

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by

Lisa Amelia Steiner III

(B.A., Swarthmore College, 1954
M.A., Radcliffe College, 1956)

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ABSTRACT

An investigation has been made of the role of the four disulfide bonds of bovine pancreatic ribonuclease in maintaining the protein in a biologically active form. Studies were carried out to determine the effect of reductive cleavage of these bonds on the ability of the enzyme to catalyze the hydrolysis of ribonucleic acid. The appearance of sulfhydryl groups was taken as evidence that reduction of the protein had occurred.

No significant reduction or loss of enzymic activity of ribonuclease could be demonstrated when the protein was treated with the reducing agents sodium or potassium borohydride in aqueous solution at room temperature, pH 8.

Thioglycolic acid was found to be an effective agent for reducing ribonuclease disulfide bonds. At room temperature, in an aqueous solution containing a large excess of thioglycolate over protein, reduction proceeded slowly at pH 8. At the end of five hours, approximately one disulfide bond was broken, with the loss of 20 per cent of the original enzyme activity. The addition of urea greatly facilitated reduction. The rate of reduction was especially rapid in solutions of urea concentration greater than 4 molar. In 8 M urea at pH 8, treatment of ribonuclease with thioglycolate resulted in the complete loss of enzymic activity in one half hour, with the simultaneous rupture of two or three disulfide bonds. Under these conditions, maximum reduction was achieved in approximately two hours, with cleavage of between three and four disulfide bonds per molecule. In the pH range from 3 to 10, rate of activity loss was most rapid at pH 10, slightly less rapid at pH 3, and reached a minimum near pH 5. The effects of pH and urea were additive in that the maximum rate of inactivation occurred at pH 10 in 8 M urea (97 per cent activity loss in 10 minutes), and the minimum rate at pH 5 in the absence of urea (20 per cent loss in 28 hours).

Inactivation was markedly inhibited by phosphate ions. A solution of protein which was 0.36 M in phosphate at pH 8 lost activity very slowly when treated with thioglycolic acid, even in the presence of 4 M urea. These findings, together with the observation of other workers that polyvalent ions such as phosphate reverse the denaturation of ribonuclease in urea, suggest that phosphate inhibits reduction by stabilizing the protein in its native configuration, whereas urea facilitates reduction by denaturing the protein.

Air oxidation of fully or partially inactivated protein resulted, in some cases, in the recovery of up to 40 per cent of the enzyme activity which had been lost as a result of reduction.

The relation between loss of activity and reduction was analyzed by correlating the data obtained in those experiments in which both the sulfhydryl concentration and the enzymic activity of samples of modified protein were determined. The experiments were carried out under a variety of conditions of pH and urea concentration. On the basis of these data, it is concluded that the inactivation of thioglycolate-treated ribonuclease is probably not a unique function of extent of reduction, but depends in part on the method by which the reduction is achieved.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

I. INTRODUCTION

A. Some Aspects of Protein Structure

The structure of a protein molecule is determined in part by the linear order of its constituent amino acids in one or more peptide chains, and in part by the characteristic manner in which these peptide chains are folded. In the following discussion, the linear arrangement of amino acids connected by peptide bonds will be referred to as the primary structure of the protein, whereas the spatial configuration or folding of the peptide chains will be known as the secondary structure. The work to be presented in this report is concerned with the effect on the enzyme ribonuclease of altering part of its secondary structure, specifically, the disulfide linkages.

Methods for investigating the primary structure of proteins were developed by Sanger (80, 83, 84), for insulin, and have been applied to other proteins including ribonuclease (8, 40). Although rather difficult experimentally, the determination of the sequence of amino acids in a single peptide chain is now a straightforward problem. No methods, however, are available for the direct determination of the secondary structure of proteins. Information about spatial configuration is usually obtained by studying those properties of the protein which are functions of molecular size and shape, such as diffusion, light scattering, sedimentation, viscosity, hydrogen ion equilibria, and optical rotation. X-ray diffraction studies of crystalline proteins show promise of providing the most detailed information on the three dimensional geometry of protein molecules.

The forces responsible for maintaining secondary structure may be divided into the strong, directed, covalent bonds, exemplified by

disulfide linkages, and the weaker, more elusive interactions such as hydrogen bonds, salt linkages, "hydrophobic" bonds and van der Waals forces which are lumped together as non-covalent bonds (47).

1. Covalent Bonds Responsible For Protein Folding

Although cross linkages through phosphate groups have been demonstrated in certain proteins by Perlmann (71), the principal covalent bonds involved in the maintenance of protein secondary structure are the disulfide bonds of cystine residues. These may link two separate peptide chains, as in insulin (80, 84) or may join two half-cystine residues of a single chain as in ribonuclease (81) and serum albumin (44). It has been suggested by Schellman (85) that disulfide bridges increase greatly the stability of the folded peptide configuration. These cross linkages may stabilize the aggregates of hydrogen bonds which are primarily responsible for protein folding, but which have little stability in themselves. On the other hand, Tanford (96) has pointed out that the disulfide cross links may interfere with the formation of "hydrophobic" bonds and salt linkages by sterically hindering the appropriate portions of the protein from approaching each other. The effect of this restraint may be to decrease the stability of the secondary structure. Although for a given protein there is no way of predicting which of these two effects will predominate, the importance of disulfide bonds to the structural and functional integrity of many proteins is illustrated by the change in physical and biological properties which frequently accompanies cleavage of these bonds. For instance, reduction of the disulfide bonds of insulin, crotoxin, and lysozyme decreases the solubility of these proteins (68). Reduction of insulin, crotoxin, and trypsin results in loss of biological activity (58, 68).

2. Non-Covalent Bonds Responsible for Protein Folding

In some proteins such as horse hemoglobin, several peptide chains may be held together in a stable state without covalent cross links between the chains (46, 72). The existence of non-covalent bonds must be invoked, therefore, to explain the stability of such molecules. In addition to cross links between peptide chains, intramolecular folding is also determined largely by non-covalent bonds. Of these, the most important is probably the hydrogen bond (65). Within proteins, hydrogen bonds exist between the carboxyl oxygen and amide nitrogen of peptide bonds (43, 69), as well as between certain side chain groups. An example of the latter is the postulated bond between a tyrosine hydroxyl and a carboxylate ion (55). Strong evidence for the existence of hydrogen bonding in proteins has been obtained by infrared spectroscopy (17). Other non-covalent intramolecular bonds which may be responsible for protein folding are "hydrophobic" bonds (attractive forces between non-polar side chains), van der Waals forces, and salt bridges between positively and negatively charged side chains (47). Although hydrogen bonds and other non-covalent bonds are weak forces individually, taken together they provide the major forces holding a protein in its folded configuration.

3. Denaturation

Denaturation may be defined as any change in the properties of a protein which is not attributable to disruption of peptide bonds. This process may be detected by a variety of physical, chemical, and biological changes in the protein, such as decrease in solubility, changes in molecular size and shape, increased reactivity of functional groups, increased susceptibility to proteolytic digestion, and loss of biological

activity (73). The denaturation of a protein need not be accompanied by all of these changes, but the occurrence of any one of them is sufficient to establish that an alteration in secondary structure has taken place. For example, urea, in concentrations which ordinarily denature proteins by disrupting hydrogen bonds, has little effect on the optical rotation of pepsin, and no effect on its proteolytic activity (47, 92). Trypsin (22) and pepsin (8) retain full enzymatic activity after partial reduction of their disulfide bonds. More complete reduction of the disulfide bonds of these two enzymes is, however, accompanied by inactivation (8, 58). Similarly, it has been reported that a preparation of partially reduced ribonuclease can be obtained which has full enzymatic activity and the same ultra-violet absorption spectrum as the native enzyme (4).

Agents which are responsible for the denaturation of proteins include those which disrupt non-covalent bonds and those which break disulfide or other covalent cross linkages. Thus, denaturation may be brought about by physical agents such as heat, freezing, and irradiation, chemical agents such as hydrogen and hydroxyl ions, organic solvents (e.g. alcohol), detergents, and amides (e.g. urea), and by reagents which split disulfide bonds, such as performic acid and thioglycolic acid. It has been found that to achieve maximum denaturation of some proteins it is necessary to use more than one denaturing agent. For example, some of the disulfide bonds of insulin resist reduction unless the protein is also treated with urea (60). Presumably, urea unmasks the disulfide bond making it available to the reducing agent (73).

Some proteins may be reversibly denatured in that they regain their activity when the denaturing agent is removed. For instance, trypsin may be boiled and rapidly cooled with no loss of activity, although at the elevated temperature it no longer digests casein (73). Kauzmann (47) has

pointed out that the reversible denaturation of proteins in urea may be a function of the disulfide cross linkages which prevent the complete unfolding of the protein molecule. Thus, serum albumin, which is reversibly denatured in urea, becomes irreversibly denatured if its cystine residues are reduced to cysteine.

B. Ribonuclease

1. Isolation and Properties

Ribonuclease was first crystallized in 1939 by Kunitz who purified it from an acidic pancreatic extract by salt fractionation (52). McDonald modified the procedure by boiling the extract to eliminate protease activity (63). The method of Kunitz and McDonald is used today in the commercial preparation of crystalline pancreatic ribonuclease.

Ribonuclease, prepared by this method, can be fractionated into two components by adsorption chromatography (62), ion exchange chromatography (36), and zone electrophoresis (74). The larger fraction is known as ribonuclease A, the smaller as ribonuclease B, but the proportion of the two varies in different preparations. The two components have similar physical and chemical properties and show the same specific enzyme activity (8). According to titrimetric studies, fractions A and B differ by only a single ionized carboxyl group (98).

The molecular weight of ribonuclease, based on its amino acid composition, is 13,700 (38). Physical studies indicate that the protein molecule is symmetrical and compact in shape (6, 14). Many cross linkages are needed to maintain such a highly folded secondary structure and these are provided both by disulfide bridges and by non-covalent forces. Ribonuclease is a remarkably stable protein. Full enzymatic

activity is recovered after incubation at room temperature for one hour between pH 1 and pH 11. Heating to 100°C. for 20 minutes at pH 5 also does not lead to loss of activity (49). This unusual stability makes the enzyme a convenient one with which to work.

2. Amino Acid Composition

The amino acid composition of ribonuclease A has been worked out by Hirs, Stein, and Moore with the method of ion exchange chromatography (37). The protein is made up of 124 amino acid residues including a relatively high proportion of basic amino acids. Eight half-cystine residues have been demonstrated (6). The isoionic point measured in dilute KCl has been reported to be 9.6 (99). There has been some controversy about the existence of sulfhydryl groups in the native enzyme. Ledoux (56, 57) reported that oxidized glutathione, parachloromercuribenzoic acid (PCMB), and a number of other agents which react with sulfhydryl groups inhibit ribonuclease activity. Zittle (104) found that treatment of ribonuclease with iodoacetic acid and iodoacetamide resulted in slow inactivation of the enzyme. However, a number of other investigators have found that incubation of ribonuclease with iodoacetate, PCMB and oxidized glutathione under mild conditions, in which these agents probably react with sulfhydryl but not with other groups on the protein, did not affect enzymatic activity (15, 16, 75). It is possible that Zittle's results may be explained by the reaction of iodoacetic acid with lysine, histidine, and tyrosine residues of the protein which occurs more slowly than the reaction with sulfhydryl groups (51). Most workers agree that there are no sulfhydryl groups in native ribonuclease and that the half-cystine residues are all linked by disulfide bridges (6).

The ribonuclease molecule consists of a single peptide chain with N-terminal lysine and C-terminal valine (5). Almost the entire amino acid sequence of the protein has been determined by the independent efforts of Hirs, Stein, and Moore (39, 40), and Anfinsen and his colleagues (8, 76). The general method used by both groups has been the isolation, analysis, and ordering of peptide fragments obtained by proteolytic digestion of the molecule. Once the amino acid sequence of a few remaining peptides is established, the primary structure of the protein will be completely known.

3. Secondary Structure

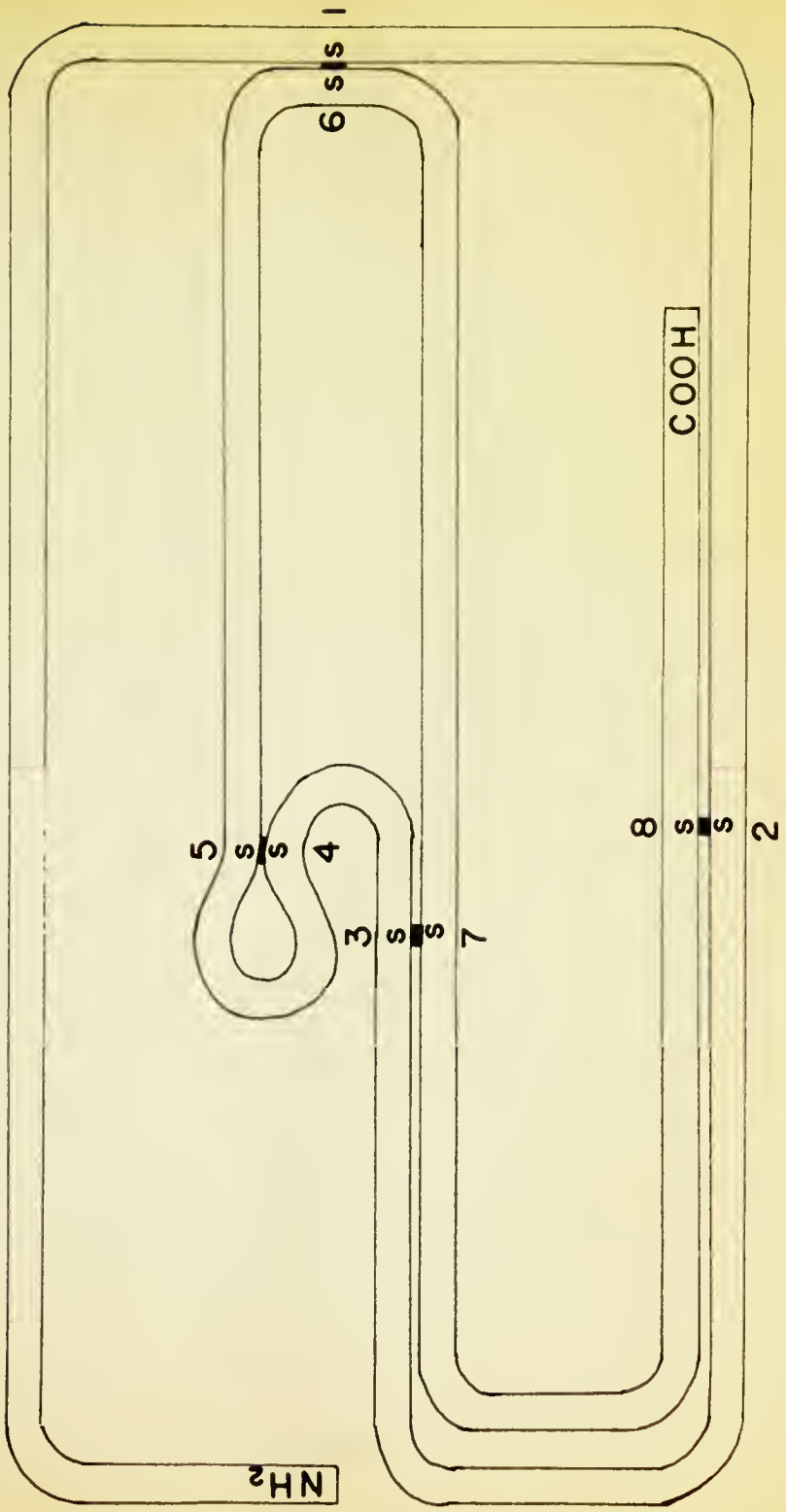
Of special interest to the present study is the location of the disulfide cross links of ribonuclease. This problem has been investigated by Ryle and Anfinsen (4, 81), and Spackman, Stein, and Moore (40, 90), using the technique of amino acid analysis of cystine-containing peptide fragments. The half-cystine residues are paired as follows: I-VI, II-VIII, III-VII, IV-V, where the roman numerals refer to the cystine residues in order, beginning at the N-terminal end of the peptide chain. Figure 1 (40) is a schematic representation of the ribonuclease molecule showing the position of the disulfide cross links.

Evidence for hydrogen bonding in ribonuclease has been obtained in a number of ways. The deuterium exchange studies of Linderstrøm-Lang and collaborators (7, 45, 59) indicated that only 72 out of 122 imide hydrogen atoms exchanged rapidly with deuterium in aqueous solution. On the other hand, all the imide hydrogen atoms exchanged freely if the disulfide bridges had been oxidized with performic acid or if the protein were dissolved in 8 M urea instead of in water. Apparently, some of the

Figure 1

Two-dimensional diagram indicating the positions of the four disulfide bonds in native ribonuclease (40). The roman numerals refer to the linear order of the half-cystine residues beginning at the N-terminal end of the amino acid chain.

FIGURE 1



RIBONUCLEASE DISULFIDE BONDS

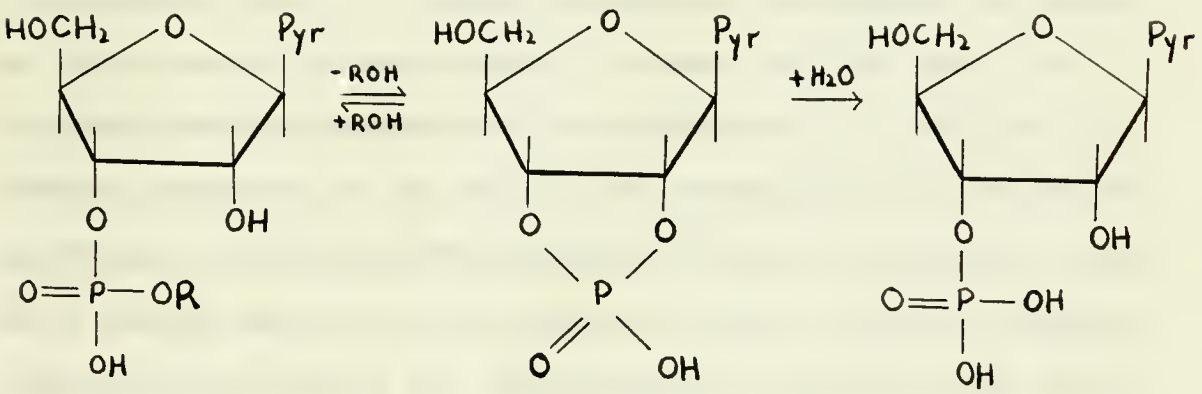
hydrogen atoms are involved in bonds which preclude free exchange. If the secondary structure is disrupted and the hydrogen bonds broken, all the hydrogen atoms become freely exchangeable. The presence of hydrogen bonding between the hydroxyls of tyrosine and carboxylate groups has been demonstrated by Shugar (89), and Tanford and collaborators (97). The failure of some of the tyrosine residues to ionize in their usual pH range probably indicates participation by these residues in hydrogen bonds.

Further evidence implicating hydrogen bonding in the maintenance of ribonuclease secondary structure has been obtained by Anfinsen and co-workers, who observed that the ultra-violet absorption, intrinsic viscosity, and optical rotation of the protein are altered when it is dissolved in 8 M urea (4). (Urea disrupts intramolecular hydrogen bonding by itself forming hydrogen bonds with appropriate groups on the protein.) Complete reversal of the observed changes may be obtained by removal of urea from the enzyme. In addition, partial reversal of these changes can be obtained by the addition of a sufficient quantity of polyvalent anions (e.g. 0.05 M phosphate) to the solution of protein in urea (4). Under these circumstances, the ultra-violet absorption reverts to that of native ribonuclease, but measurements of optical rotation and intrinsic viscosity indicate that the protein in the presence of urea and phosphate remains in a partially unfolded state. Anfinsen had previously noted that ribonuclease in 8 M urea had full enzymatic activity (7). On the basis of the above observations he postulated that ribonucleic acid may be capable of partially refolding the protein in the presence of 8 M urea, the substrate acting in this respect like other polyvalent anions. That the protein seems to be partly unfolded in the presence of urea and phosphate, and, presumably, also in

the presence of urea and ribonucleic acid, may mean that catalytic activity is not necessarily destroyed by a limited degree of modification of the secondary structure.

4. Enzymatic Properties

Pancreatic ribonuclease degrades ribonucleic acid in two stages. First, the phosphodiester bond linking the 3' position of a pyrimidine nucleoside to the 5' position of the next nucleoside in the RNA chain is split with the formation of the pyrimidine nucleoside 2',3'-phosphate. This step is relatively rapid. The cyclic pyrimidine nucleoside phosphate is then slowly hydrolyzed by the enzyme to yield the nucleoside 3'-phosphate (26).



Phosphodiester of pyrimidine nucleoside

Pyrimidine nucleoside 2',3'-phosphate

Pyrimidine nucleoside 3'-phosphate

Assays for ribonuclease activity usually involve a spectrophotometric measurement of either the first or the second stage of the catalytic process.

The optimum pH for ribonuclease activity is close to 7.7 both for the first (52) and second stage (10). The enzymic activity is markedly dependent on the ionic strength of the medium (29). Certain ions such

as Cu^{++}, Zn^{++} (15, 104) and some other polyvalent ions such as heparin (78) and desoxyribosenucleic acid (34) inhibit activity. Products of ribonucleic acid digestion have also been found to act as inhibitors (15, 19).

5. Modification of Ribonuclease

The relationship between structure and biological activity of ribonuclease has been studied by a number of workers. Richards (77) has shown that a change in primary structure resulting from the cleavage by the enzyme subtilisin of a single peptide bond 20 amino acid residues from the N-terminal end of the chain, with subsequent separation of the fragments, yields two peptide chains neither of which has significant enzymatic activity. Recombination of the two fractions results in restoration of activity. Limited digestion of ribonuclease with pepsin at pH 1.8 results in the splitting of a single bond four amino acid residues from the C-terminal end and in inactivation of the enzyme (4). However, degradation of the protein with carboxypeptidase which has been pretreated with diisopropylfluorophosphate removes the C-terminal valine and a variable amount of the two adjacent residues, serine and alanine, with no loss of activity (4). This would seem to implicate the removal of the aspartic acid residue, which is fourth from the end, in the inactivation brought about by pepsin.

It has also been demonstrated that certain chemical modifications of the enzyme result in changes in activity. Klee and Richards (48, 49) have shown that guanidination of nine of the ten lysine residues of ribonuclease does not affect enzymatic activity, whereas guanidination of the tenth lysine results in complete inactivation. Taborsky (95) has shown that treatment of ribonuclease with diphosphoimidazole and Ca^{++} under mild alkaline conditions results in phosphorylation of certain

epsilon amino groups of lysine and complete loss of activity. Inactivation is also brought about by photooxidation of histidine residues as shown by Weil and Seibles (102).

C. Reactions of Protein Disulfide Bonds

The disruption of disulfide bonds is frequently an important part of an investigation of protein structure. The purpose of such a step is twofold: first, the clarification of the role of these disulfide bridges in the structure of the protein, and second, the facilitation of enzymatic degradation of the protein which is a consequence of cleavage of the disulfide cross links. It has been found by many investigators that rupture of the disulfide linkages may be difficult to accomplish without preliminary unfolding of the protein to unmask these bonds. In the case of performic acid oxidation of the disulfide bonds, the oxidizing agent itself is sufficiently powerful to disrupt the secondary structure of the protein and thereby react with all the disulfide groups. However, with milder agents such as mercaptans, sulfites, or borohydrides, it is usually necessary to treat the protein with urea or some other unfolding agent if cleavage of all the disulfide groups is to be attained.

1. Performic Acid Oxidation

Rupture of the disulfide bridges of proteins by performic acid oxidation results in the conversion of cystinyl groups to cysteic acid residues. This procedure, however, destroys tryptophan residues and quantitatively converts methionine to the sulfone (67). Both insulin, which contains no tryptophan or methionine, and ribonuclease, which has methionine but no tryptophan, have been oxidized with performic acid (6, 82). Oxidized ribonuclease is enzymically inactive (3).

2. Reactions with Sulfite

The undesirable side effects of performic acid oxidation have led to a search for other means of breaking protein disulfide bonds. Bailey and Swan have studied the reaction of proteins with sulfite (9, 94). In the presence of cupric ions, cystinyl residues are converted to S-sulfocysteyl groups. This reaction shows a high degree of specificity, but its usefulness is presumably limited because of difficulties with the analytical procedures needed to determine the extent of reaction (67). Recently, however, the sulfite reaction has been applied by Pechère et al. (70) to the cleavage of the disulfide bonds of trypsinogen and chymotrypsinogen. These workers, as well as Bailey and Swan, carried out the reaction in 8 M urea. The extent of the reaction was determined by polarography and incorporation of S^{35} sulfite. Although the formation of S-sulfotrypsinogen and S-sulfochymotrypsinogen involved some modification of the secondary structure of the native proteins, gross molecular integrity was retained and the authors feel that this method may have general applicability to the structural analysis of proteins.

3. Reduction

Another method used to break disulfide bridges is the reduction of these bonds under conditions sufficiently mild to minimize other changes in protein structure. Whenever some but not all of the disulfide bonds in a protein are broken, it is desirable to measure the specificity as well as the extent of reduction. One may then determine whether partial reduction is the result of random cleavage of the disulfide bonds or whether certain bonds are broken more easily than others. Lindley (60), for example, found that the intrachain disulfide bond of the A chain of

insulin was reduced preferentially over the interchain disulfide bridges. Information about the sequence and speed of reduction of disulfide bonds may be obtained by studying the homogeneity of the partially reduced protein. Fraenkel-Conrat (24) showed that a sample of partially reduced lysozyme was composed of two clearly defined fractions, one fraction consisting of almost completely reduced molecules and the other of unreacted molecules. Such a finding indicated that the reduction of the first one or two disulfide bonds in a given molecule was a rate limiting step which was followed by the rapid reduction of the remaining disulfide linkages of that molecule.

Various agents have been used in attempts to reduce protein disulfide bonds. Early studies, particularly with insulin, employed such methods as catalytic hydrogenation (2, 25) and reaction with cyanides (12, 18). These methods proved unsatisfactory and have been supplanted by the use of mercaptans and, very recently, borohydrides.

a. Sodium borohydride. Sodium borohydride, as described by Schlesinger (86), is a stable reducing agent which can be used conveniently in aqueous solution. It has been used to reduce the disulfide bonds of lipoic acid (32), wool (28), insulin (20), and bovine serum albumin (91).

Recently, Moore and his colleagues have used sodium borohydride to break the disulfide bonds of ribonuclease (67). Following the reduction, the protein was alkylated by treatment with iodoacetic acid. The extent and specificity of reduction were determined by amperometric titration of sulfhydryl groups, Alexander's n-ethylmaleimide method for sulfhydryl groups (1), and analysis of the amino acid composition of hydrolysates of the alkylated reduced protein. It was found that complete reduction

could be achieved relatively rapidly in aqueous solution at 51°C. At 41°C., however, the reaction went to completion quickly only in solutions of urea concentration greater than 4 molar. Amino acid analysis indicated that the specificity of both the reduction and the alkylation was fairly high, with recovery of over 90 per cent of all the amino acids of the native protein except for cystine. Cystine was recovered, in good yield, as the reduced, alkylated derivative, S-carboxymethylcysteine. These investigators have also been able to reduce the cystine residues of trypsinogen, lysozyme, chymotrypsinogen, and β-lactoglobulin under the same conditions.

b. Mercaptans. Since 1925 when Hopkins (42) found that the disulfide groups of proteins could be reduced by treatment with cysteine, glutathione, or thioglycolic acid, a number of mercaptans have been used to bring about the reduction of a variety of proteins. Insulin (60, 101), lysozyme (24), and lens protein (18), are among the proteins whose disulfide bridges have been reduced by thioglycolate, cysteine, mercaptoethanol, and other thiols. Most reductions have been carried out in slightly alkaline solution, but the reaction has also been carried out at pH 5, and in one report, some reduction was achieved at pH 2 (93). The reaction has frequently been run in 8 M urea. Several workers have found thioglycolic acid to be the most effective mercaptan for the reduction of the disulfide bonds of some proteins (21, 64, 88).

The reduction of protein disulfide bonds by mercaptolysis has the important advantage of specificity. However, the extent of reduction cannot be determined directly by sulfhydryl assay unless the mercaptan reducing agent, which is present in considerable excess, is separated

completely from the protein. This may be accomplished by dialysis, precipitation of the protein, and similar procedures. An alternative is to stabilize the protein sulfhydryl groups (e.g. by alkylation) and then to submit the protein to amino acid analysis. The number of alkylated cysteine residues is determined in this manner.

The mechanism of the reduction of disulfide bonds by simple mercaptans has been studied by Lamfrom and Nielsen (54) using as a model the reduction of cystine by thioglycolic acid. These investigators have shown that the mercaptolysis proceeds by the following exchange reaction:

$$\begin{aligned} RSH &\rightleftharpoons RS^- + H^+ \\ RS^- + CSSC &\rightleftharpoons CSSR + CS^- \\ CSSR + RS^- &\rightleftharpoons RSSR + CS^- \\ CS^- + H^+ &\rightleftharpoons CSH \end{aligned}$$

where CSSC = L-cystine; CSH = L-cysteine; RSH = thioglycolic acid; RSSR = dithiodiglycolic acid; RSSC = "mixed" disulfide. The reactive form of the mercaptan in this scheme is the mercaptide ion. Comparing their findings for simple disulfides with the reactions of protein disulfide groups, Lamfrom and Nielson noted that it is relatively more difficult to achieve complete reduction of the protein. This necessitates the use of urea in protein systems. The authors suggest that the incomplete reduction in the absence of urea may not be due to masked disulfide bonds, as has usually been thought, but to an intermolecular reshuffling of disulfide bonds catalyzed by the mercaptan reducing agent. New disulfide links formed in this way may be resistant to reduction because of stabilizing forces such as hydrogen bonds, hydrophobic bonds, etc..

Evidence for similar disulfide exchange reactions has been obtained by Ryle and Sanger (79). These investigators found that the exchange reaction between L-cystine and NN'-bis-2:4-dinitrophenyl-L-cystine was catalyzed by thiols in neutral or alkaline solution, but inhibited by

thiols in acid solution. It was suggested that the reason for the catalysis in alkaline solution was the rapid rate of formation of mercaptide ions in such solutions. However, the reason for the inhibition in acid solution was not understood.

Anfinsen and his colleagues have recently carried out an extensive study on the reduction of the disulfide bonds of ribonuclease by thioglycolic acid, and the effect of this reduction on enzymatic activity (4, 87, 88). Extent of reduction was determined by titration of protein sulfhydryl groups (after removal of thioglycolate) with PCMB and by amino acid analysis of alkylated reduced protein. The two methods gave comparable results. It was found that thioglycolate brought about the rapid reduction of ribonuclease in 8 M urea at pH values near 8. However, in the absence of urea, only two of the four disulfide bridges were reduced. Measurements of the activity of the partially reduced enzyme led to the conclusion that one or perhaps two of the disulfide bridges were not essential for activity. Examination of the data presented, however, reveals that activity is lost more or less simultaneously with rupture of disulfide bonds and that the conclusion that any of these bonds is not essential for activity is open to question. These workers have also been able to obtain some regeneration of enzymatic activity following reoxidation of sulfhydryl groups with molecular oxygen. They have some evidence which suggests that under these conditions there may be partial reformation of the original disulfide bonds.

The effect of reduction of disulfide bonds on biological activity has been examined for several other proteins. Fraenkel-Conrat et al. (24) found that reduction of the disulfide links of lysozyme in 8 M urea is

accompanied by rapid disappearance of enzymatic activity. In the case of insulin, it has been found that reduction of one third of the disulfide bonds results in complete or nearly complete inactivation (23, 103). In the light of Lindley's finding of preferential reduction of the intrachain disulfide bond (60), these results would seem to indicate that cleavage of this one bond leads to loss of biological activity. One attempt to restore the activity of insulin by reoxidation of the protein resulted in failure (23).

The data reported in this thesis are concerned with the effects of reduction of the disulfide groups of ribonuclease on the activity of this enzyme. At the time these studies were begun, there were no reports in the literature on this subject. The papers which have subsequently appeared, notably those of Anfinsen and Moore, have been discussed in the preceeding paragraphs.

II. MATERIALS AND METHODS

Ribonuclease. Crystalline bovine pancreatic ribonuclease, Lot No. 381-059, supplied by Armour and Co., was used without further purification. This preparation consists of ribonuclease A and ribonuclease B in the approximate ratio of nine to one.

Spectrophotometric measurements. A Beckman model D.U. spectrophotometer was used throughout these experiments. All readings were carried out at room temperature.

Hydrogen ion concentration. pH measurements were carried out at room temperature with a Photovolt, model 125, pH meter which was calibrated with standard buffers prepared according to the recommendations of the National Bureau of Standards.

Reducing agents. Sodium and potassium borohydrides were supplied by Metal Hydrides, Inc., Beverly, Mass. Thioglycolic acid was obtained from Matheson, Coleman and Bell Division, Matheson Co..

Other materials will be listed in the sections below. Unless otherwise indicated, all chemicals mentioned were used as supplied by the manufacturer, without further purification.

A. Sulfhydryl Determinations

1. Amperometric Titration

Sulfhydryl groups were measured, on occasion, by amperometric titration according to the method of Kolthoff and Harris (50). This method is based upon the reaction of an ammoniacal solution of silver nitrate with the mercaptan according to the equation,

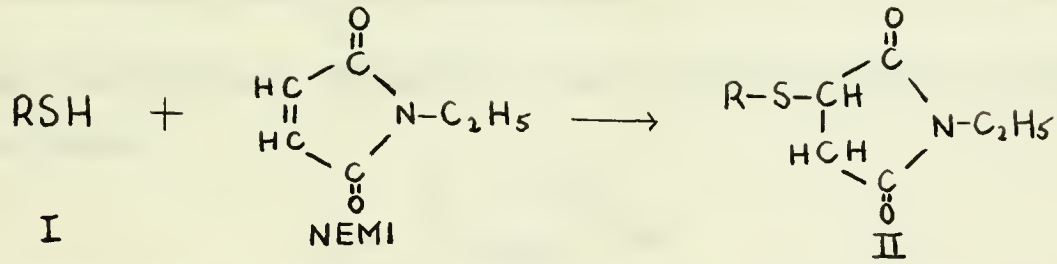
$$Ag(NH_3)_2^+ + RSH \longrightarrow RSAg + NH_4^+ + NH_3$$

2. Nitroprusside Reaction

Occasionally, sulfhydryl groups were determined colorimetrically by the nitroprusside reaction. The method used was that described by Grunert and Phillips (31).

3. N-ethylmaleimide Assay

N-ethylmaleimide (NEMI) was purchased from Schwarz Laboratories, Mount Vernon, N.Y. This reagent reacts stoichiometrically with mercaptans according to the following equation,



Aqueous solutions of NEMI have an absorption maximum near 300 millimicrons. The mercaptan addition products have very low absorption, in general, at this wavelength. The change in optical density at 300 millimicrons that occurs during this reaction has been made the basis of a sulfhydryl assay procedure by Alexander (1). The change in optical density is assumed to represent the change in NEMI concentration and thus serves as a measure of the amount of mercaptan added. A 0.001 M solution of NEMI in 0.1 M phosphate buffer at pH 6.8 has an optical density of 0.620 at 300 millimicrons (1,30). Mercaptans added to this solution to give concentrations between 0.0001 and 0.0009 M produce an optical density change proportional to the concentration of mercaptan. The composition of the solutions used in a typical assay is given in Table 1.

Table 1

PROTOCOL FOR TYPICAL NEMI ASSAY

Solution	Initial NEMI	Phosphate Buffer pH 6.8	R-SH unknown (0.1 to 0.9 mM)	Measured Optical Density (300 mμ)
1	1.0 mM	0.1 M	x mM	A_1
2	1.0 mM	0.1 M	None	A_2
3	None	0.1 M	x mM	A_3

The concentration of the unknown mercaptan solution was then calculated as follows,

$$x = \frac{A_2 - (A_1 - A_3)}{0.620} \text{ mM}$$

A_2 was measured in each case to compensate for errors in dilution or minor degrees of deterioration of the NEMI solution, although it never varied much from 0.620 in optical density. The assumption in the use of this method is that compounds I and II (see equation on p. 19) have the same molar absorption at 300 millimicrons, and therefore that any difference between initial and final absorption is due only to a change in the concentration of free NEMI. This assumption is questionable. A more general approach is the following.

Small aliquots of NEMI are added to a given solution of mercaptan. The absorption of the solution is measured after each addition and plotted as a function of the total amount of NEMI added. Assume the following symbolism:

A = measured absorption of the mixture
a_1 = molar absorbancy of I
a_2 = " " " II
a_3 = " " " NEMI

c_1^o = initial concentration of I
c = concentration of total NEMI added

During first additions, the mercaptan is in excess, the added NEMI reacts quantitatively, and only compounds I and II are present in the solution.

$$A = a_1 (c_1^o - c) + a_2 c \qquad (c < c_1^o)$$

$$= a_1 c_1^o + (a_2 - a_1) c$$

After the end point of the titration the mercaptan has reacted quantitatively and only compound II and NEMI are present in solution.

$$A = a_2 c_1^o + a_3 (c - c_1^o) \qquad (c > c_1^o)$$

$$= (a_2 - a_3) c_1^o + a_3 c$$

At the endpoint,

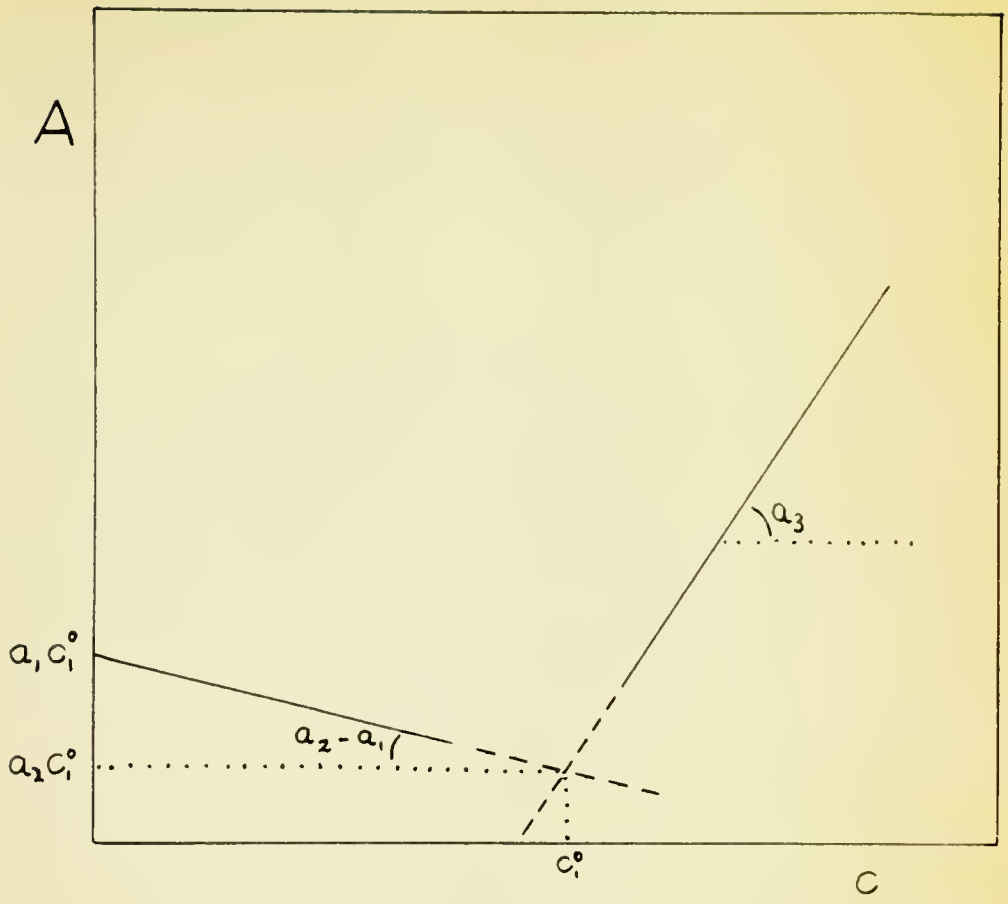
$$A = a_2 c_1^o$$

$$c = c_1^o$$

Since A is a linear function of c both in the region of sulfhydryl excess and in that of NEMI excess, the intersection of two straight lines gives the equivalence point as shown in the diagram (Figure 2).

Although it was thought that this modification of the assay described by Alexander had some theoretical advantages, it was found that in the measurement of samples of the reduced protein, the two methods agreed very closely. This corresponds to the observation that the initial portion of the titration curve (region of sulfhydryl excess) was usually horizontal, indicating that compounds I and II have approximately the same optical density at 300 millimicrons.

FIGURE 2



NEMI ASSAY

TITRATION METHOD

One further variation was introduced when it was found that reduced ribonuclease, which is less soluble than the native enzyme, often precipitated out of solution during the NEMI assay. Since the reduced protein was much more soluble in urea than in water, the assay was set up with four or eight molar urea in all solutions. The method of collection of the reduced protein was also modified so as to keep it in solution. (See Section II-B-2.)

When the sulfhydryl concentration of a given solution of thioglycolic acid was determined by assaying both in water and in 8 M urea, the assay in urea indicated a sulfhydryl content about 10 per cent lower than that obtained in water. The reason for this difference was not apparent. With few exceptions, and these clearly indicated, the NEMI assay was used for all sulfhydryl determinations reported in this paper. This assay has the distinct advantage that the sulfhydryl content of an unknown solution can be determined directly by optical density measurements, and need not be determined by comparison with standard mercaptan solutions which are difficult to store without loss of sulfhydryl groups.

In agreement with the findings of Gregory (30), distilled water solutions of NEMI were found not to change in optical density during a one month period of storage in the cold, although at pH 6.8 the reagent was unstable. NEMI was generally prepared as a 0.004 M stock solution, and, immediately before assay, was brought to pH 6.8 with phosphate buffer. The reaction between NEMI and simple mercaptans such as cysteine or thioglycolic acid was found to be very rapid, the entire detectable change in optical density occurring within the first minute. When the solutions used in the assay were made 8 M in urea, the buffer was adjusted to pH 6.8 after the addition of urea. It was found, however, that variation in the pH from 6.5 to 7.3 had little effect on the assay.

Before attempting to measure the sulfhydryl groups of reduced ribonuclease, the assay was tested on a protein of known sulfhydryl content. A solution of aldolase, prepared according to the method of Taylor et al. (100), was obtained from Dr. S. Rieder. It was found that this particular preparation of aldolase contained, in two separate determinations, 22 and 23 equivalents of sulfhydryl per mole of protein. Benesch et al. (11), using the method of amperometric titration, have reported that aldolase contains 20 reactive sulfhydryl groups per molecule and an additional 9 which become available upon denaturation with 8 M urea.

B. Separation of Protein from Reducing Agent

1. Precipitation of Protein

Repeated washing and precipitation is a standard procedure for desalting a protein. This method was applied to the separation of ribonuclease from thioglycolic acid.

The results of precipitation and washing of native ribonuclease with 95 per cent ethanol were compared with those obtained by precipitation with 5 per cent trichloroacetic acid followed by washing with ether. Although fully active protein was recovered in each instance, the use of alcohol gave a higher percentage of recovery of protein. Therefore, this method was chosen for the precipitation of the reduced enzyme. A typical reduction, followed by alcohol precipitation of the protein is described below.

A 1.0 per cent solution of ribonuclease was incubated at pH 8.5 with 0.3 M thioglycolic acid, 0.05 M tris [tris(hydroxymethyl)aminomethane] buffer, and 0.5×10^{-4} M ethylenediaminetetraacetic acid (EDTA). At the end of the period of reduction, two volumes of 2 M, pH 5 acetate buffer were added to the solution. (It was thought initially that acidification

to pH 5 would stop the reduction reaction, although subsequent work has shown this not to be the case.) This was followed by the addition of five volumes of a solution 95 per cent in ethanol, 0.06 M in pH 5 acetate buffer, and 1.0×10^{-4} M in EDTA. The precipitate obtained after centrifugation was washed twice with 95 per cent ethanol and centrifuged after each washing. The final precipitate was dissolved in 0.02 M, pH 5 acetate buffer. Occasionally, the precipitate could not be dissolved unless urea was added to the solution of buffer. The sulfhydryl concentration of the solution obtained was determined by the NEMI assay and the protein concentration by the Folin-Lowry assay. The supernatant solutions from the washings were also analyzed for sulfhydryl concentration.

Although thioglycolic acid could be eliminated virtually completely by washing twice with an excess of ethanol, only very small amounts of protein sulfhydryl could be demonstrated. It was thought that complete separation of the reducing agent from the protein might be followed by air oxidation of the reduced ribonuclease. The procedure was modified, therefore, so that a small measured amount of thioglycolic acid was allowed to remain with the protein in the final precipitate. It was hoped that the presence of small amounts of reducing agent would prevent the oxidation of protein sulfhydryl groups.

After precipitation with an excess of ethanol, the protein was centrifuged but not washed, and the sulfhydryl concentrations of both the precipitate and supernate determined. The concentration of thioglycolic acid in the precipitate was assumed to be the same as that in the supernate. The concentration of protein sulfhydryl was then determined by subtracting the concentration of thioglycolic acid from the

total thiol concentration of the precipitate. The volume of the precipitate was obtained by weighing and measuring the specific gravity of this fraction. The total number of ribonuclease sulfhydryl groups could then be determined.

Several difficulties appeared in the application of this method. Since the thioglycolate-treated protein was less soluble than the native enzyme, solutions containing reduced protein were often turbid or frankly cloudy and could not be assayed accurately for sulfhydryl content by the NEMI method. The presence of thioglycolic acid in the solutions of reduced protein also decreased the sensitivity of the measurement of protein sulfhydryl groups. The procedure was modified again, therefore, so that after the initial alcohol precipitation, the protein was centrifuged, then washed once and centrifuged again. Now only a small amount of thioglycolic acid remained in the final ribonuclease solution. This modification increased the sensitivity of the assay for protein sulfhydryl groups, but also increased the risk of incurring air oxidation of these groups. In addition, the turbidity of solutions containing large amounts of reduced protein continued to interfere with spectrophotometric procedures. Although it was possible to detect some reduction of the protein under these conditions, it became clear that a more satisfactory method for the separation of ribonuclease from reducing agent was needed.

2. Anion Exchange

Thioglycolate was separated from ribonuclease by the method of anion exchange using Amberlite IRA 400 resin, 20-50 mesh (Rohm and Haas). The resin was washed by repeated cycling with 1 N NaOH and 1 N HCl, and then converted to the chloride or acetate cycle by washing with 1 N hydrochloric or 1 N acetic acid.

In a typical experiment, one ml. of a solution containing less than 350 mM of thioglycolate was added to a column approximately 1.2 cm. in diameter, and containing five to eight ml. of settled resin. The solution was allowed to run into the resin at the rate of about one drop per second, and the rate of flow then decreased to one drop every twenty or thirty seconds. The column was then washed with distilled water or solutions of 4 M or 8 M urea. The effluent was collected in graduated centrifuge tubes or cylinders.

It was necessary to establish that all the thioglycolate used to reduce the enzyme could be removed in one passage through the column. One ml. of a solution containing twice the concentration of thioglycolate usually employed in the reduction of the enzyme was added to the column and the effluent tested for sulfhydryl groups. At pH 9, all the thioglycolate was exchanged by the resin in the chloride cycle. At pH 3 and pH 5, however, approximately one half to one per cent of the thioglycolate added appeared in the effluent. This amount of mercaptan would be sufficient to introduce a large error in the protein sulfhydryl determination. When the resin was converted to the acetate cycle, however, the thioglycolate was completely exchanged at pH 3 and pH 5 as well as at pH 9.

An additional check on the efficiency of the column in removing thioglycolate was the demonstration that following a period of incubation at room temperature, if a mixture of protein and thioglycolate were added to the resin (chloride cycle, pH 8), two successive effluent fractions contained proportionate amounts of protein and sulfhydryl. This meant that the sulfhydryl groups which appeared in the effluent had come through with the protein. It seemed probable, therefore, that this sulfhydryl was actually part of the protein molecule.

Further evidence that the column selectively removed thioglycolate was afforded by the following experiment. Ribonuclease and thioglycolate were mixed under conditions in which no reduction of the protein occurred. The reaction mixture was passed through the column (chloride cycle, pH 8). No sulfhydryl groups could be demonstrated in the effluent.

It was found that native ribonuclease could be recovered from the column in good yield without loss of activity. Because the reduced protein was less soluble than the native enzyme, on some occasions protein which had been treated with thioglycolic acid precipitated out of the effluent solution, making impossible the spectrophotometric assay for sulfhydryl groups. If solutions of urea, instead of distilled water, were used to wash the column, the reduced protein remained in solution. The NEMI assay was then carried out with all solutions having the same concentration of urea as was used in washing the column.

Since reoxidation of the sulfhydryl groups of reduced ribonuclease may occur very rapidly in air, the column effluent was usually collected under nitrogen. The exposure to oxygen was reduced to the few seconds needed to add the effluent to the NEMI solutions for sulfhydryl assay.

C. Ribonuclease Activity Assays

1. Anfinsen Assay

This assay, a modification of the one described by Anfinsen (6), depends on the determination of the number of nucleotides liberated during the enzymatic hydrolysis of ribonucleic acid. It measures the first stage of ribonuclease activity.

The ribonucleic acid substrate was prepared as follows. A ten per cent solution of Schwarz yeast nucleic acid in 0.1 M sodium chloride was precipitated with one fifth volume of glacial acetic acid and three volumes of 95 per cent ethanol. The resulting precipitate was washed

approximately four times with 75 per cent ethanol in 0.02 M sodium chloride. The precipitate was then dissolved in sufficient 0.1 M acetate buffer at pH 5 to make the concentration of the final solution one half to one per cent in ribonucleic acid. If the optical density of the blank in the assay exceeded 0.200 at 260 millimicrons, the nucleic acid was reprecipitated and washed repeatedly until the blank fell below this value.

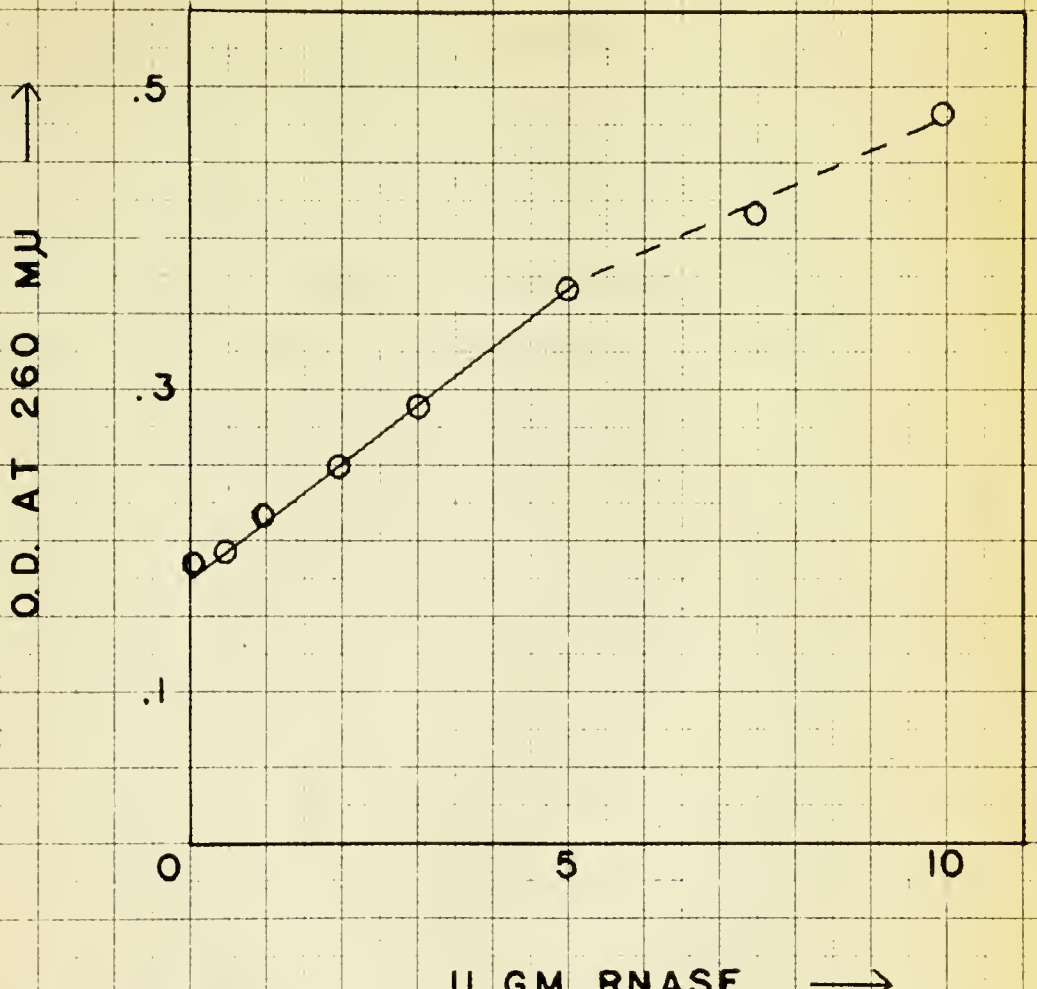
A solution containing less than 10 micrograms of ribonuclease in five to ten microliters was incubated with one ml. of ribonucleic acid substrate at 25°C. for 15 minutes. The reaction was stopped by precipitation with one ml. of a solution containing 0.5 per cent uranyl acetate and 2.5 per cent trichloracetic acid (41). The resulting precipitate was allowed to settle for five minutes, and then centrifuged. The optical density of a one to eleven dilution of the supernatant was determined at 260 millimicrons. The reagent blank contained all solutions except the enzyme.

The variation in measured ribonuclease concentration between duplicate samples was almost always less than plus or minus 5 per cent, and usually less than plus or minus 2 per cent of the average value. Samples were always run in duplicate, and a standard curve was included with each assay. A linear relationship was observed between ribonuclease concentration and optical density, the extent of linearity depending on the particular substrate preparation employed. A representative standard curve is reproduced in Figure 3.

Standard solutions of ribonuclease containing at least 10 mg. of protein per 10 ml. were stored in the refrigerator. It was shown that a solution containing 3 mg. of protein per 10 ml. lost no activity when kept in the cold or at room temperature for two days.

The term "per cent activity" means the measured activity of the

FIGURE 3



STANDARD RNASE ASSAY

(ANFINSEN)

modified enzyme expressed as the per cent of that expected for the same weight of native enzyme.

2. Kunitz Assay

This assay is based on the observation that the enzymatic degradation of ribonucleic acid results in a shift in its ultra-violet absorption spectrum towards shorter wave lengths. The change in spectrum is accompanied by a decrease in absorption near 300 milimicrons. Measurement of the rate of decrease in absorbancy at 300 millimicrons of a ribonucleic acid solution in the presence of ribonuclease was used by Kunitz to determine enzyme concentration. This assay, like Anfinsen's, depends upon the first stage of ribonuclease action.

A substrate solution was prepared by diluting the ribonucleic acid solution used in the Anfinsen assay with 0.1 M acetate buffer at pH 5.0. The dilution was adjusted so that the substrate had an optical density at 300 millimicrons of approximately 0.80. The usual dilution was one to fifteen. One ml. of substrate was added to a quartz cuvette, and the optical density at 300 millimicrons recorded. A solution containing less than 10 micrograms of ribonuclease in five to ten microliters was then added directly to the substrate in the cuvette, and the resulting solution mixed by vigorously pipetting up and down with a micropipette. The optical density at 300 millimicrons of this solution was followed for a total of three to ten minutes.

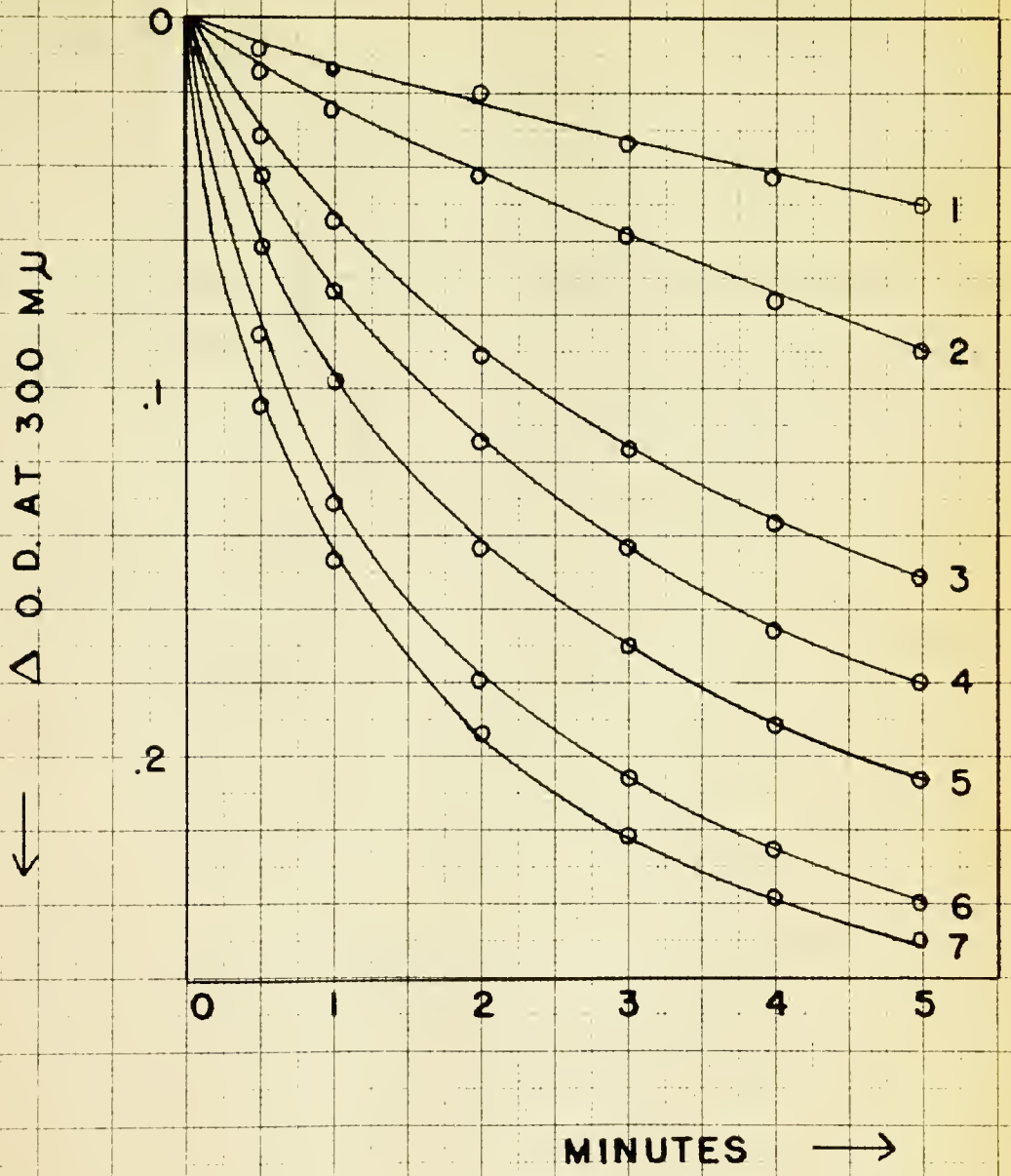
The enzyme concentration of the solution being assayed can be determined in a number of different ways. The simplest procedure is to fit the curve for the unknown solution to a series of standard curves such as those in Figure 4. The unknown ribonuclease concentration is then

Figure 4

Kunitz assays were performed as described in the text. The amounts of ribonuclease used were as follows:

Curve No.	Micrograms Ribonuclease
1	0.5
2	1.0
3	2.0
4	3.0
5	5.0
6	7.5
7	10.0

FIGURE 4



KUNITZ ASSAY STANDARDS

obtained by direct comparison. This method proved to be inconvenient, however, because a great deal of time is needed to obtain such a series of standard curves.

Another method for determining enzyme concentration involves the assumption that this reaction obeys Michaelis-Menten enzyme kinetics according to the equation,

$$\frac{-d(S)}{dt} = \frac{K_3(E)(S)}{(S) + K_m}$$

where (S) is the substrate concentration at time t, (E) is the total enzyme concentration (a constant), and K_3 and K_m are constants. From the integrated form of the equation,

$$K_m \log \frac{(S_o)}{(S)} + (S_o)-(S) = K_3 (E)t$$

it can be seen that the enzyme concentration is inversely proportional to the time required to reach a given extent of reaction. ((S_o) is the substrate concentration at t = 0.) The concentration of enzyme in an unknown solution can then be determined by comparing its assay curve with that of a single standard solution. This was the method used to calculate the ribonuclease activity in most of the Kunitz assays reported in this paper.

In order to apply the Michaelis-Menten equation, it is necessary to fulfill the assumption of the theory that there be a large molar excess of substrate over enzyme. In these assays, therefore, as large a concentration of substrate was used as was compatible with accurate reading on the spectrophotometer, and as little enzyme as would yield a significant optical density change (greater than 0.1 optical density units) in the substrate. The one to fifteen dilution of stock ribonucleic acid satisfied the condition for the substrate since its optical density

$$\frac{\quad}{\quad} \quad \frac{\quad}{\quad}$$

at 300 millimicrons was approximately 0.8. The standard amount of enzyme used was usually three micrograms.

Kunitz stated that this reaction follows first order kinetics (53), but in the present study this relationship did not obtain. Indeed, the assay curves indicated product inhibition rather than a linear relationship between the logarithm of the substrate concentration and time. It has been demonstrated by other workers that the products of hydrolysis of ribonucleic acid inhibit ribonuclease activity (19).

A modification of the Kunitz assay was introduced in which the enzyme was assayed in the presence of urea. The ribonucleic acid substrate was prepared as before except that it was made eight molar in urea. It was thought that the activity of the reduced protein, when compared with that of the native enzyme might be different in the presence of urea than in its absence. Although such a study was eventually attempted, it was complicated by the effect of the reducing agent on the partially reduced protein in the presence of eight molar urea. (See Section III-B-1-C.) This complication may be avoided if the reducing agent is separated from the protein prior to assay.

One practical difficulty with the Kunitz assay arises from temperature changes. Since the optical density was followed in a spectrophotometer which had no temperature control, much variation was observed in the optical density changes obtained with a standard enzyme preparation as the instrument changed in temperature. It was found that this variation was minimized if the spectrophotometer was allowed to warm up for at least one hour before use. In addition, standards were included with all assays to permit compensation for fluctuations in temperature.

The assay procedure was analyzed by plotting the product of enzyme concentration and time against change in optical density at 300 millimicrons

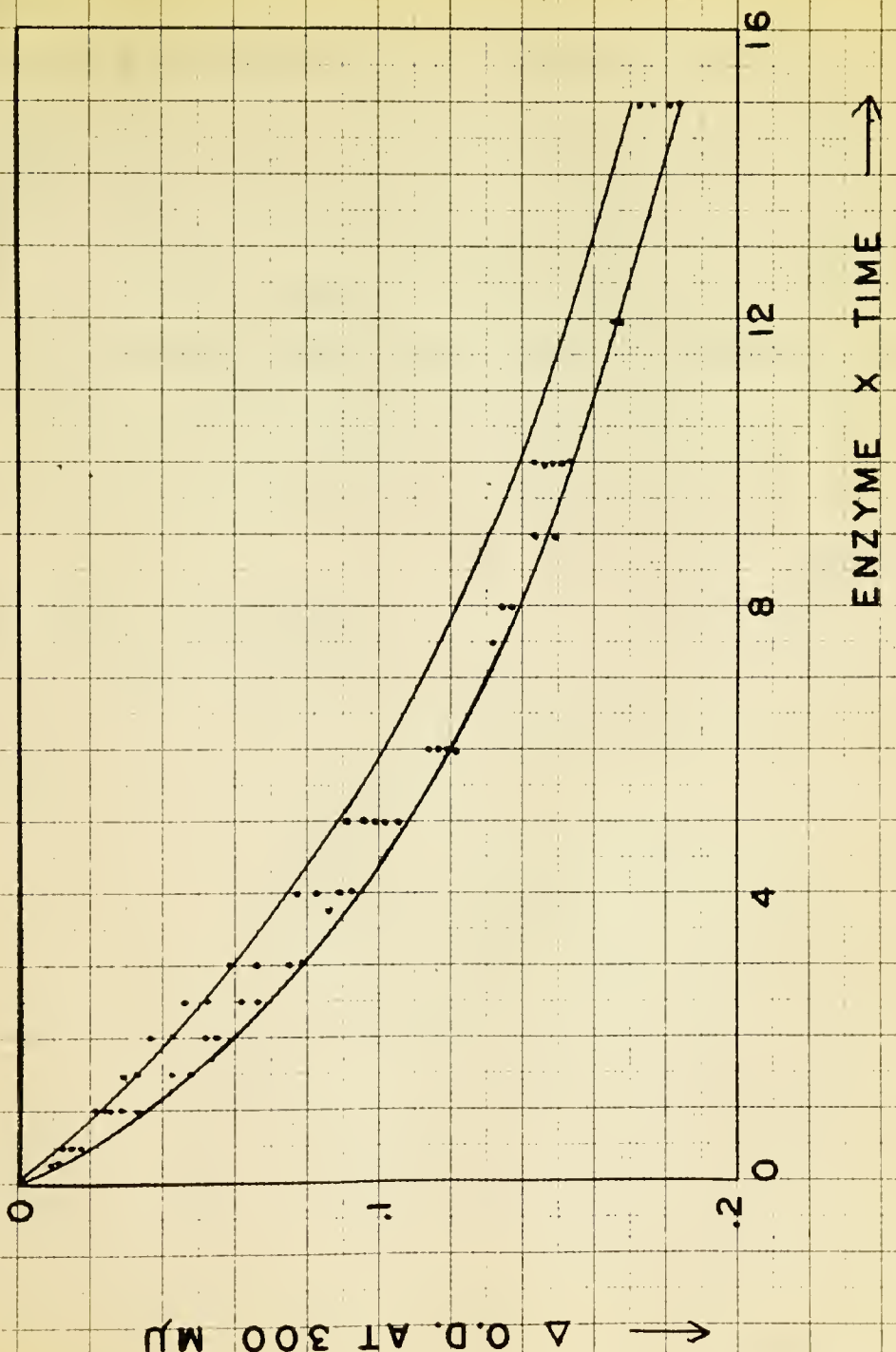
In this way, all the information in one or more series of standard curves can be assembled in a single curve. Such a curve is illustrated in Figure 5, which is a composite of two series of standard curves obtained on different days. The two lines in the figure are drawn so that virtually all the points fall between them. These curves, therefore, represent the extreme limits of variation of the assay. For a given change in optical density in excess of 0.10, the variation in the enzyme-time product was less than plus or minus 12 per cent of the average value, and under these conditions this was the extreme error for the assay. The assay could be made more accurate by diluting the unknown solution so that its concentration was close to that of the standard ribonuclease sample. The error then approached the difference between assay of duplicate solutions, approximately plus or minus 5 per cent of the average value. Samples were always run in duplicate.

Although up to 10 micrograms of native ribonuclease could be measured in the Kunitz assay with the accuracy indicated in the preceeding discussion, certain difficulties were encountered in the measurement of the activity of the reduced enzyme. It was found that the addition of 5 or 6 micrograms of 75 per cent reduced enzyme resulted in an increase in the optical density at 300 millimicrons of the ribonucleic acid substrate during the first few minutes of the assay, followed by a slower increase for the remainder of the assay period. The addition of the same amount of 50 per cent reduced enzyme to the substrate brought about an initial increase in optical density lasting perhaps one minute, followed by some decrease in absorbancy such as usually indicated enzyme activity. This effect was not studied quantitatively, but the initial increase in optical density seemed roughly proportional to the amount of enzyme added.

Figure 5

The product of enzyme in micrograms and time in minutes is plotted against change in optical density at 300 millimicrons. The two solid lines indicate the maximum limits of error of the assay. The points are taken from two sets of standard curves obtained on different days.

FIGURE 5


0
.1
.2
0
4
8
12
16
Δ O.D. AT 300 MμM
ENZYME X TIME
ENZYME - TIME PRODUCT PLOT

A similar observation was made by Klee (48) while measuring the activity of guanidinated ribonuclease in the Kunitz assay.

The initial increase in optical density was not observed if the protein sample assayed had not been extensively inactivated or if less than two micrograms of protein were used. Despite the possibility that small degrees of this effect may have introduced some error into the measurement of activity of all samples of partially reduced ribonuclease, it was assumed that this error was not large in those cases in which an initial increase in optical density was not detected. That this assumption was probably correct was demonstrated by the finding that samples of partially reduced enzyme had approximately the same activity when measured by both the Kunitz and Anfinsen assays.

D. Protein Determinations

1. Folin-Lowry Method

Folin-Ciocalteu phenol reagent was obtained from Eimer and Amend, Fisher Scientific Company.

The method of Lowry et al. (61) was used for almost all protein determinations. This method depends on the reduction of the phosphomolybdic-phosphotungstic (Folin phenol) reagent by protein which has been treated with an alkaline solution of copper. It was found unnecessary to titrate the phenol reagent as described, and it was simply diluted with one volume of water prior to use. Standards of native ribonuclease were compared in each assay with the unknown solutions of reduced enzyme. The optical density of the standard solutions at 700 millimicrons was linear with respect to protein concentration up to 100 micrograms of enzyme.

It was found that thioglycolic acid reacted with the phenol reagent,

the optical density after assay treatment of a 0.001 M solution of thioglycolate being approximately 0.16. The protein determination, therefore, could be carried out only after separation of the ribonuclease from the reducing agent.

Since the color produced by thioglycolic acid in this reaction may be due to the binding of copper by the sulfhydryl groups of the mercaptan, it was thought that a similar binding by the thiol groups of the reduced protein might introduce a significant error into the protein measurement. However, it was determined that if the sulfhydryl content of the protein produced an amount of color equal to that produced by an equivalent amount of thioglycolic acid, the error introduced into the protein assay would be negligible.

It was shown that the color developed in the Folin assay by ribonuclease which had been denatured in 8 M urea was approximately equal to the color produced by an equal amount of native protein.

2. Optical Density Measurements

The molecular weight of ribonuclease, based on its amino acid composition, is 13,700. A 0.1 per cent solution of ribonuclease has, according to an average of extinction coefficients given in the literature, an optical density of 0.720 at 280 millimicrons (35, 95). The water content of the ribonuclease used was approximately ten per cent. This was verified by measurements of optical density which yielded an average absorbance at 280 millimicrons of 0.650 for a 0.1 per cent solution (uncorrected for water content) of protein. In calculating protein weights, compensation was made for the ten per cent water content.

The absorbance of the reduced protein at 280 millimicrons was taken as a rough check on the Folin protein assay. Values for the protein

concentration of extensively reduced enzyme obtained by optical density measurements were approximately ten per cent lower than those obtained by means of the Folin assay. This discrepancy corresponds to the magnitude and direction of the spectral shift at 280 millimicrons observed when ribonuclease is denatured in 8 M urea, indicating that the reduced protein is undoubtedly denatured.

III. EXPERIMENTAL RESULTS

A. Reduction of Disulfide Bonds with Sodium and Potassium Borohydride

The reduction of protein disulfide bonds with sodium or potassium borohydride offers the distinct advantage that the reducing agents do not interfere with subsequent determination of sulfhydryl groups. The borohydrides are relatively stable in alkaline solution. Heat, or the addition of acid causes them to decompose rapidly with the production of hydrogen gas and various boron complexes. It has been noted that the formation of these complexes may make the analysis of reduction products difficult (27).

1. Reduction of Cystine

Before attempting the reduction of ribonuclease with sodium and potassium borohydride, these reagents were incubated with cystine and reduction of the disulfide bond of this amino acid measured. Cystine, and as a control cysteine, were treated with a 10 to 100 fold molar excess of sodium or potassium borohydride in alkaline solution. The extent of reduction of cystine to cysteine was determined by amperometric titration, the nitroprusside reaction, and paper chromatography.

Amperometric titration. Although the sulfhydryl groups of cysteine could be measured without difficulty in the absence of sodium or potassium borohydride, it was found that the presence of these agents interfered with the amperometric titration of the thiol groups. Despite the lability of the borohydrides in acid solution, it was not possible to eliminate this interference by acidifying the solution of mercaptan and borohydride. It was concluded that the borohydrides or their breakdown

products interfered in some unknown way with the reaction of sulfhydryl groups with the silver ion, and this method for measuring reduction of sulfhydryl groups was abandoned.

Nitroprusside reaction. As was the case with amperometric titration, it was found that the borohydrides interfered with the determination of sulfhydryl groups by the nitroprusside method. The presence of 20 micromoles of sodium or potassium borohydride suppressed almost entirely the development of color of 1 micromole of cysteine. The addition to a solution of cysteine of an acidified solution of potassium borohydride or of the borate ion alone also resulted in some interference with color development of the thiol.

With the use of this assay it was at times possible to demonstrate some reduction of cystine to cysteine. The results were never sufficiently reproducible, however, to make the method practical for quantitative work.

Paper chromatography. Reduction of cystine to cysteine by sodium borohydride was demonstrated by ascending paper chromatography with Whatman No. 1 filter paper and a solvent of water-saturated phenol (100 g. phenol: 25 ml. water) (33).

A solution of 0.003 M cystine was treated with 0.06 M sodium borohydride in the presence of 0.006 M EDTA. Following the incubation period, the solution was acidified to destroy the borohydride. Oxidation of the sulfhydryl groups was prevented by treating solutions containing cysteine or reduced cystine with 0.02 M NEMI. Solutions containing a theoretical maximum of 0.1 microequivalent of sulfhydryl were then spotted on the paper.

The chromatogram indicating reduction of cystine to cysteine is reproduced in Figure 6.

Figure 6

Chromatogram illustrating the reduction of cystine to cysteine by sodium borohydride.

A solution of 0.003 M cystine was made 0.06 M in sodium borohydride and 0.006 M in EDTA. After the incubation period, the solution was acidified by adding one fourth volume of 1 N H Cl, and then made 0.02 M in NEMI. Control solutions were included as indicated. Solutions containing a theoretical maximum of 0.1 microequivalent of sulfhydryl were spotted on Whatman No. 1 filter paper and chromatographed in a solvent of water-saturated phenol.

Abbreviations: L-cystine, CSSC; L-cysteine, CSH; N-ethylmaleimide, NEMI; Sodium borohydride, $NaBH_4$.

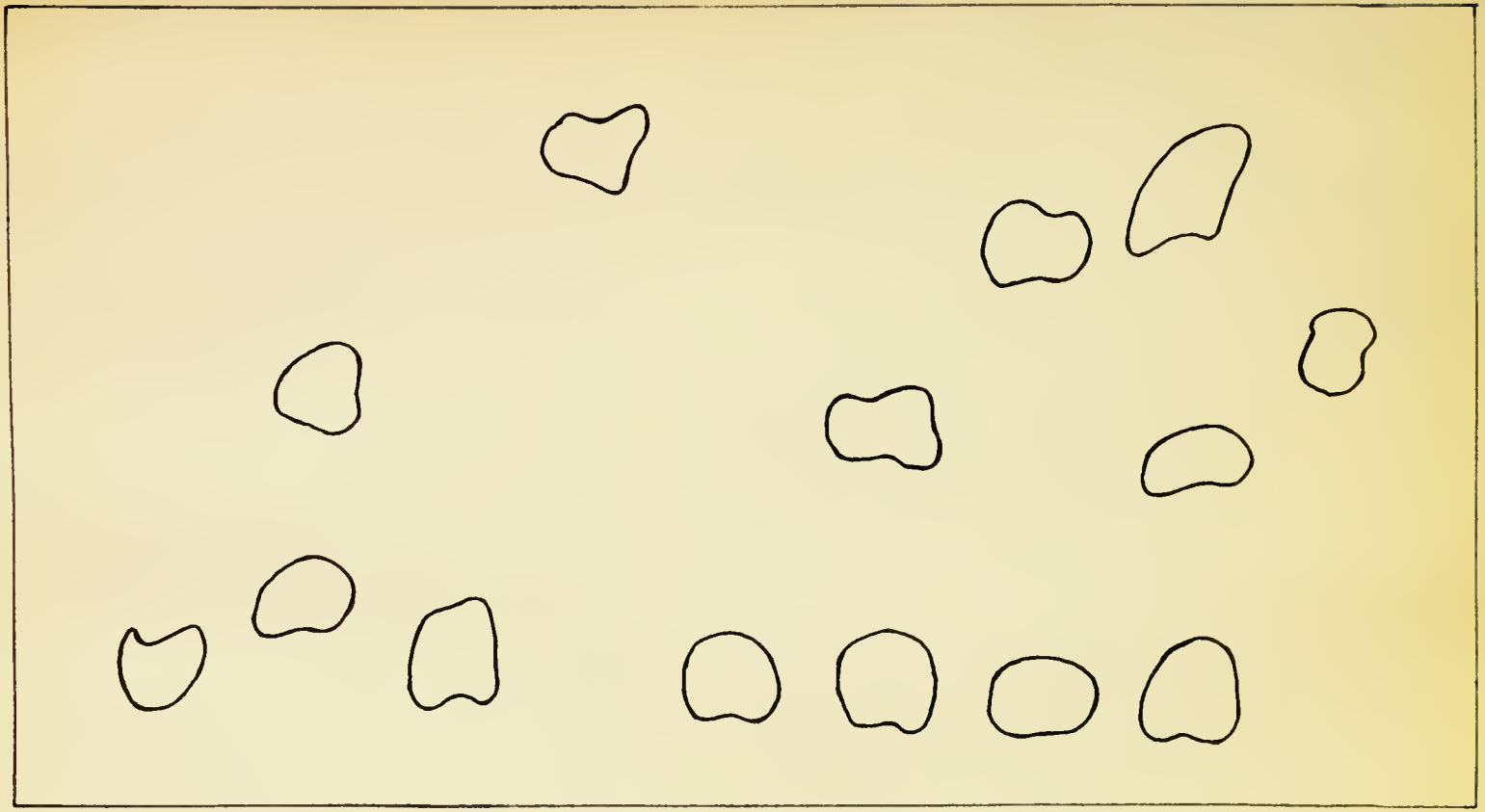


FIGURE 6

CHROMATOGRAPHY OF REDUCED CYSTINE

2. Reduction of Ribonuclease

Despite the difficulties with the sulfhydryl assays associated with the use of the borohydrides, several attempts were made to reduce ribonuclease with these agents. In each case, a solution approximately 1 per cent in ribonuclease was treated with 0.01 M sodium or potassium borohydride at pH 8. The nitroprusside reaction was used to measure sulfhydryl groups and the Anfinsen assay to determine enzyme activity. No detectable sulfhydryl groups were found after incubation of the protein with the reducing agent for periods of forty minutes and three days. There was no loss of activity at the end of two hours of incubation with sodium borohydride.

These results are similar to those reported recently by Moore et al. (67). This group of investigators found that significant reduction of ribonuclease with sodium borohydride could only be demonstrated at high temperatures or in strong urea solutions.

B. Reduction of Ribonuclease with Thioglycolic Acid

Although it had been demonstrated that the disulfide bonds of cystine could be reduced by sodium borohydride, the interference of this agent with sulfhydryl assay procedures made its use impractical and led to trials with other reducing agents. Mercaptoethylamine and β-mercaptoethanol were used on one occasion, but the reducing agent which was used for virtually all the work reported here was thioglycolic acid. All experiments were carried out at room temperature.

Evidence that the disulfide bonds of ribonuclease had been reduced by thioglycolic acid was obtained in several ways. The different types of evidence are listed below and each will then be taken up in detail in the following sections.

Loss of Enzymatic Activity. Loss of activity means that some change in the structure of the protein must have occurred. Since the expected effect of treatment with thioglycolic acid is reduction of the disulfide bonds of the molecule, loss of activity is usually taken as evidence that such reduction has taken place. There are other ways, however, in which treatment with thioglycolic acid might, under certain circumstances, result in loss of enzymatic activity. For instance, with relatively low concentrations of thioglycolate, disulfide exchange with polymerization of protein molecules at the disulfide sites might occur, leading to loss of activity without production of sulfhydryl groups. Some activity might be lost as a result of a non-specific denaturing action of the reducing agent.

It is, therefore, a potentially misleading, although a common, practice to refer to protein which has been inactivated as a result of treatment with a reducing agent as "reduced protein," even though reduction per se has not been demonstrated. This practice has, in general, been followed in this report, but it is to be understood that the terms "reduction" and "inactivation" are not necessarily synonymous. Wherever such usage might lead to confusion it has been avoided, and distinction between the two terms has been made.

Demonstration of Sulfhydryl Groups. Short of finding cysteine residues in a hydrolysate of the protein, the demonstration of sulfhydryl groups on the ribonuclease molecule is the most direct evidence that reductive cleavage of the cystine disulfide bonds has taken place.

Change in Chromatographic Behavior. As in the case of loss of activity, change in chromatographic behavior is evidence that a structural modification of the protein has been brought about, but not necessarily that disulfide bonds have been reduced.

Change in Solubility. Changes in physical properties such as solubility also indicate that there has been some modification of protein structure.

1. Loss of Enzymatic Activity

Aliquot volumes for activity assay were usually taken directly from the mixture of ribonuclease and thioglycolic acid. When a sulfhydryl determination was carried out, the thioglycolate-free protein solution obtained for sulfhydryl assay was also assayed for activity It was shown that native enzyme lost none of its activity as a result of precipitation with alcohol or passage through ion exchange resin.

Ribonuclease, when incubated with thioglycolic acid, lost activity at a rate influenced by the urea concentration, pH, and thioglycolate concentration of the solution in which the reduction took place.

a. Urea Concentration. It was noticed early in the course of this work that ribonuclease, when treated with a large excess of thioglycolic acid at pH 8, lost activity very slowly, approximately half the activity remaining at the end of 24 hours. On the other hand, when the reduction was carried out under exactly the same conditions except for the presence of 8 M urea, inactivation occurred much more rapidly. This effect was studied in a number of experiments in which the reduction proceeded in solutions of varying urea concentration. One such experiment is described below.

To several solutions of 0.09 per cent ribonuclease in 0.02 M tris buffer at pH 8.0, thioglycolate, as the sodium salt, was added to give a concentration of 0.09 M. The urea concentration was varied from 0 to

8 M.[1] Enzymatic activity was followed for 24 hours. As indicated in Figure 7, loss of activity occurred more quickly in solutions containing urea than in those without urea. Inactivation was especially rapid in those solutions which were greater than 4 M in urea. In 8 M urea, only 10 per cent of the original activity remained after 18 minutes, and at the end of 25 minutes, the enzyme was completely inactive. Control solutions of ribonuclease in 0.02 M tris buffer, pH 8, and 8 M in urea lost no activity during the experimental period.

The rapid inactivation of ribonuclease in concentrated solutions of urea is probably related to the denaturing effect of the amide. Unfolding of the protein molecule in the presence of urea may expose otherwise hidden disulfide bonds to the action of the reducing agent. It was mentioned in Section I-B-3, that Anfinsen has shown that the addition of a sufficient quantity of polyvalent anions such as phosphate to solutions of ribonuclease in concentrated urea reverses, at least partially, the physical signs which indicate unfolding of the protein. It was thought, therefore, that the addition of such anions to solutions of ribonuclease in urea and thioglycolate might prevent the unfolding of the protein and hence suppress reduction of the disulfide bonds. The effect of phosphate ions on the inactivation of ribonuclease was studied in the following experiment.

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[1] In general, when describing experiments in which ribonuclease was incubated with a number of other reagents, the hydrogen ion concentration of the buffer is the only pH mentioned. It is to be understood that either all the components of the final solution were adjusted to the pH of the buffer prior to mixing, or the solution was adjusted to this pH after mixing the components. However, in all experiments with urea, the pH mentioned refers to the hydrogen concentration of the solution before the urea was added. Although the addition of the amide led to some change in the pH of the solution as measured by the glass electrode, no attempt was made to correct this by readjusting the pH after addition of the urea. The magnitude of the change in measured pH brought about by adding the urea was at most 0.5 pH units in the alkaline direction.

Figure 7

The inactivation of ribonuclease (RNase) by thioglycolic acid (TGA) in various concentrations of urea. Per cent activity is plotted against time with urea concentration as parameter.

The conditions of the experiment were: 0.09 per cent ribonuclease, 0.09 M sodium thioglycolate, 0.02 M tris buffer, and pH 8.0. The urea concentrations were 0, 2, 4, 5, 6, and 8 M. Enzyme activity was followed for 24 hours.

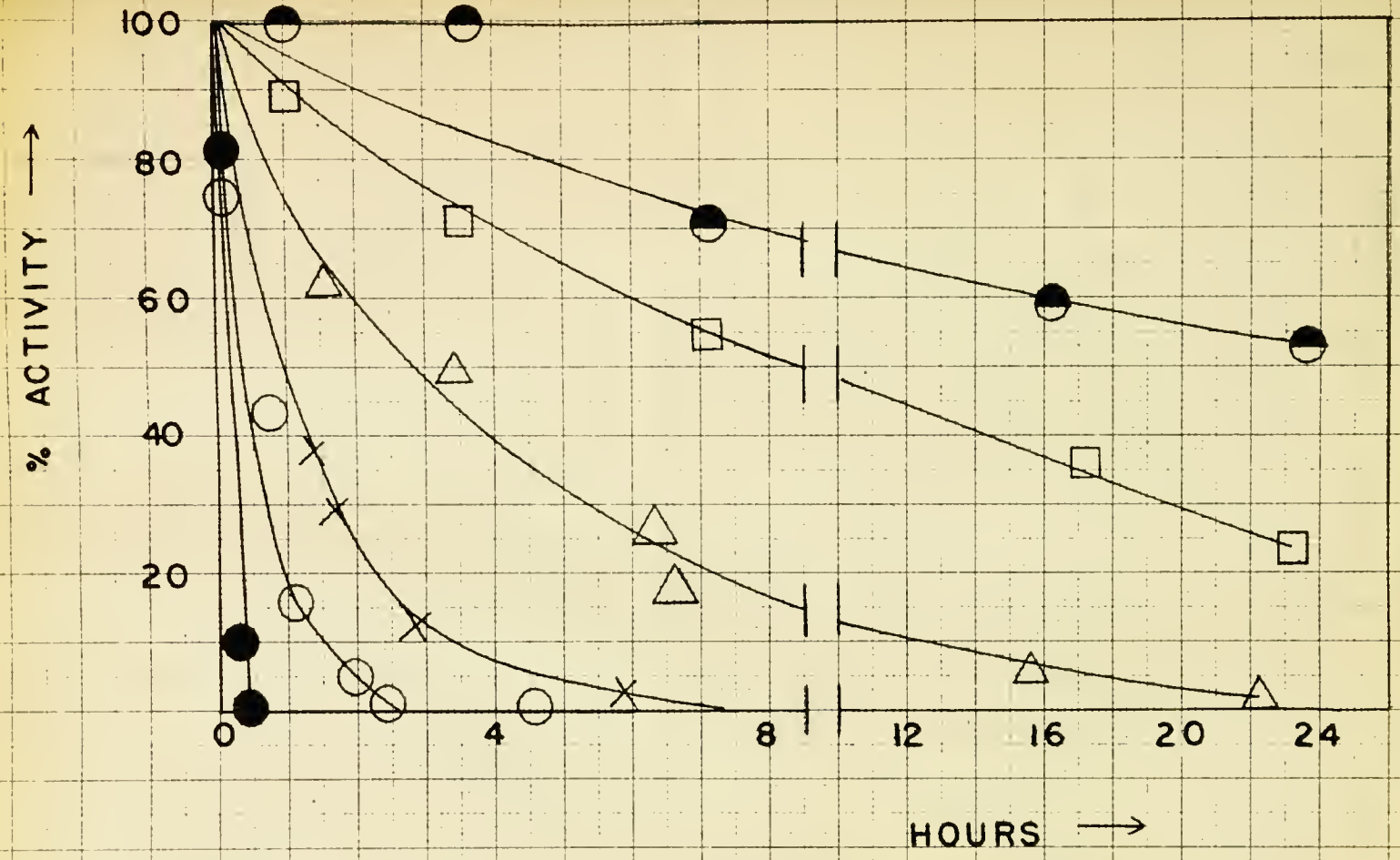


FIGURE 7

EFFECT OF UREA CONCENTRATION ON INACTIVATION OF RNASE BY TGA

0 M UREA
2 M "
4 M "
5 M UREA
6 M "
8 M "

Solutions of 0.1 per cent ribonuclease in 0.02 M tris buffer at pH 8.0 were treated with 0.1 M thioglycolic acid, 4.3 M urea, and 0.36 M potassium phosphate in the combinations indicated in Table 2. The per cent activities of these solutions during the 14 hour experimental period are summarized in this table. The time course of activity for solutions 1, 2, 3, and 4 is also illustrated in Figure 8.

The solutions containing phosphate (1 and 3) showed much less loss of activity both in the presence and in the absence of urea than did those containing no phosphate (2 and 4). Solutions 5, 6, 7, and 8 sustained no significant loss of activity during the experimental period. Since the phosphate ions suppressed the inactivation of ribonuclease regardless of the presence of urea, these anions cannot be considered specific antagonists of urea, but probably have a general action in stabilizing the protein in its native or folded state.

In summary, the information which has been obtained on the role of urea and phosphate in the inactivation of ribonuclease by thioglycolic acid indicates that significant reduction of the protein disulfide bonds will not occur without some degree of unfolding of the molecule. Since urea denatures the protein, whereas phosphate stabilizes it in its native configuration, reduction, and consequent inactivation, are facilitated by the amide, but inhibited by the polyvalent anions.

b. Hydrogen Ion Concentration. As indicated in Section I-C-3 the reduction of the disulfide bonds of proteins, including ribonuclease, has usually been carried out in alkaline solution at pH values near 8 or 9. One investigator showed that the rate of inactivation of insulin increased with rising pH over the range pH 6 to 8. The reducing agents used were cysteine and thioglycolic acid (103).

In the present study, the effect of hydrogen ion concentration on the

Table 2

THE EFFECT OF PHOSPHATE AND UREA ON THE

INACTIVATION OF RIBONUCLEASE BY THIOGLYCOLIC ACID

SOLUTION	UREA	PO_4	TGA	PER CENT ACTIVITY		
				13 min.	$3\frac{1}{2}$ hrs.	$14\frac{1}{2}$ hrs.
1	4.3 M	0.36	0.1 M	99	90	71
2	4.3 M	-	0.1 M	96	49	15
3	-	0.36	0.1 M	99	89	85
4	-	-	0.1 M	98	72	53
5	4.3 M	0.36	-	101	104	106
6	4.3 M	-	-	100	91	100
7	-	0.36	-	100	101	94
8	-	-	-	100	96	92

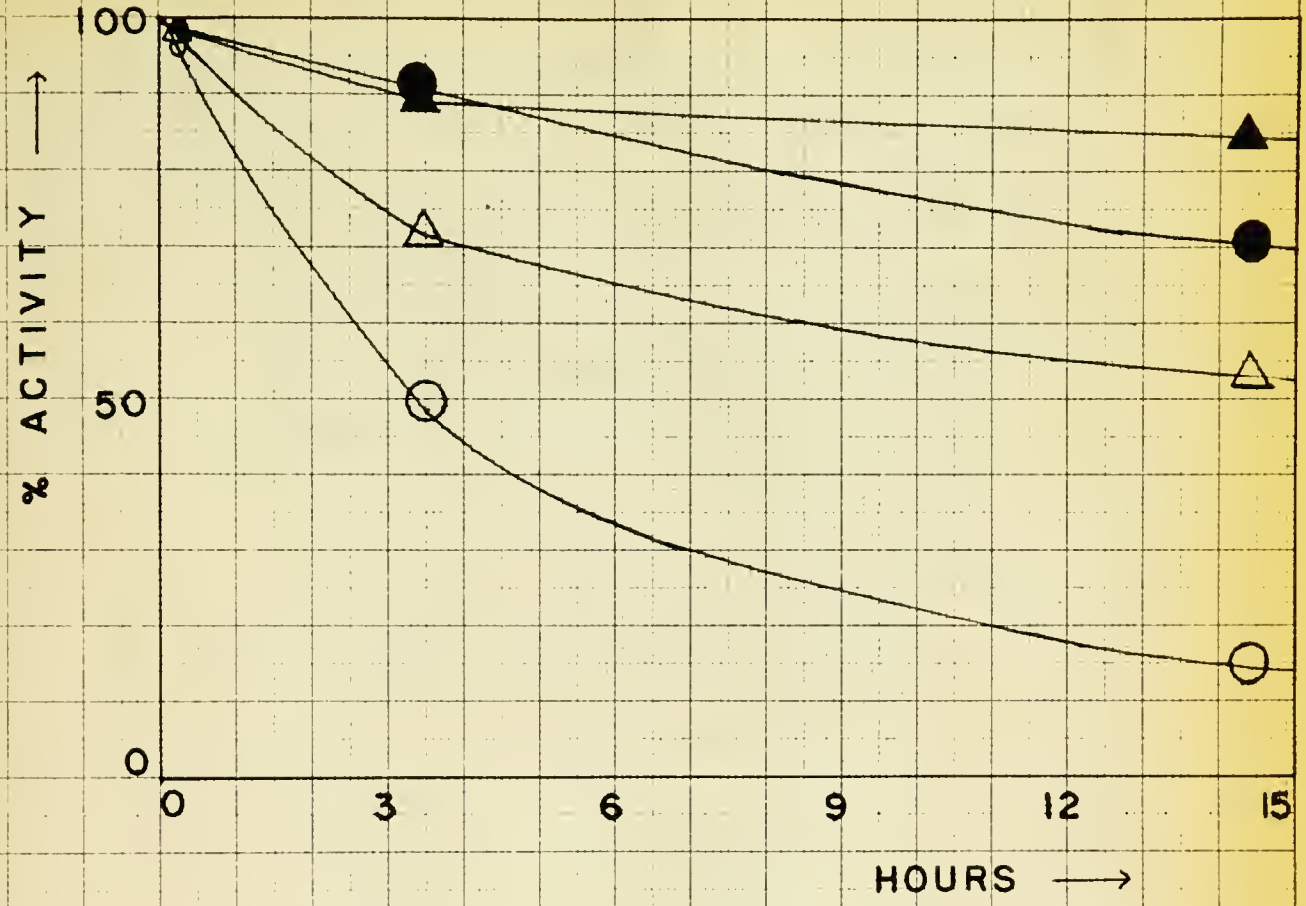
Figure 8

The effect of phosphate and urea on the inactivation of ribonuclease (RNase) by thioglycolic acid (TGA). Per cent activity is plotted against time.

Solutions of 0.1 per cent ribonuclease in 0.02 M tris buffer at pH 8.0 were treated with urea, phosphate, and thioglycolate in the concentrations indicated below.

Solution	UREA	PO_4	TGA
1	4.3 M	0.36 M	0.1 M
2	4.3 M		0.1 M
3		0.36 M	0.1 M
4			0.1 M

FIGURE 8



1 ● TGA + PO_4 + UREA

2 ○ TGA + UREA

3 ▲ TGA + PO_4

4 △ TGA

EFFECT OF UREA AND PO_4 ON

INACTIVATION OF RNASE BY TGA

inactivation of ribonuclease by thioglycolic acid was tested over the pH range from 3 to 10. In one experiment, a solution of 0.09 per cent ribonuclease in 0.02 M buffer was treated with 0.07 M thioglycolic acid in the presence of 4 M urea. The pH values tested, together with the buffers employed at each pH are indicated in Table 3. Because of the possibility of specific ion effects, two different buffers were used at several pH values. The listing of two buffers next to a specific pH value means that two samples, each containing one of these two buffers were included in the experiment. Except for tris, all buffers were present as the sodium salt. Tris was used as the chloride.

Table 3

pH VALUES AND BUFFERS USED IN TESTING THE EFFECT OF pH ON INACTIVATION OF RIBONUCLEASE BY TGA

pH	Buffer
3	Citrate
5	Citrate, Acetate
7	Barbital
8	Barbital, Tris
9	Tris, Glycine
10	Glycine

The results of this experiment are summarized in Figures 9 and 10. Inactivation occurred slowly at pH 5, but more rapidly as the pH was changed in either the acid or the alkaline direction. During the first 19 minutes of the reaction, one third of the initial activity was lost at pH 3, and two thirds at pH 10. No significant inactivation occurred during this period at pH 5, 7, or 8. At the end of 25 hours, there was extensive inactivation at all pH values tested with the exception of pH 5 and 7. The extent of the reaction was independent of the particular buffer

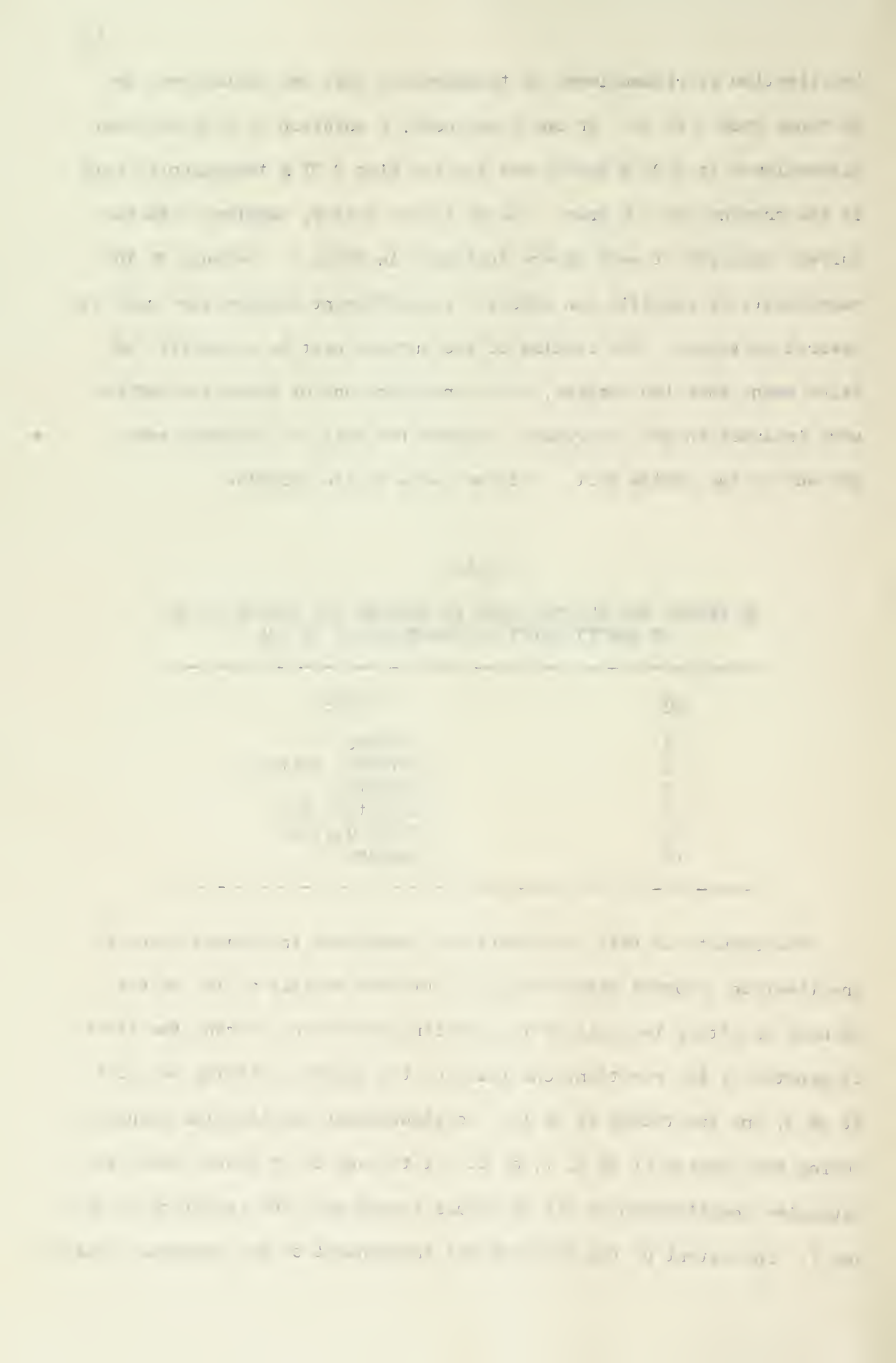


Figure 9

The effect of pH on the inactivation of ribonuclease (RNase) by thioglycolic acid (TGA). Per cent activity is plotted against time with pH as parameter.

The conditions of the experiment were: 0.09 per cent ribonuclease, 0.07 M thioglycolic acid, and 4 M urea. The pH values tested were pH 3, 5, 7, 8, 9, and 10. The buffers (concentration 0.02 M) used at each hydrogen ion concentration are listed in Table 3.

Except at pH 5, whenever two buffers were used at a single pH value, the average of the two per cent activities obtained is plotted. The per cent activities for pH 5, citrate (5 c) and pH 5, acetate (5 a) are plotted separately.

Figure 10

The effect of pH on the inactivation of ribonuclease (RNase) by thioglycolic acid (TGA). Per cent activity is plotted against pH with time as parameter.

The conditions of the experiment were as described above for Figure 9. Whenever two buffers were used at a single pH value, the average of the two per cent activities obtained is taken as the ordinate.

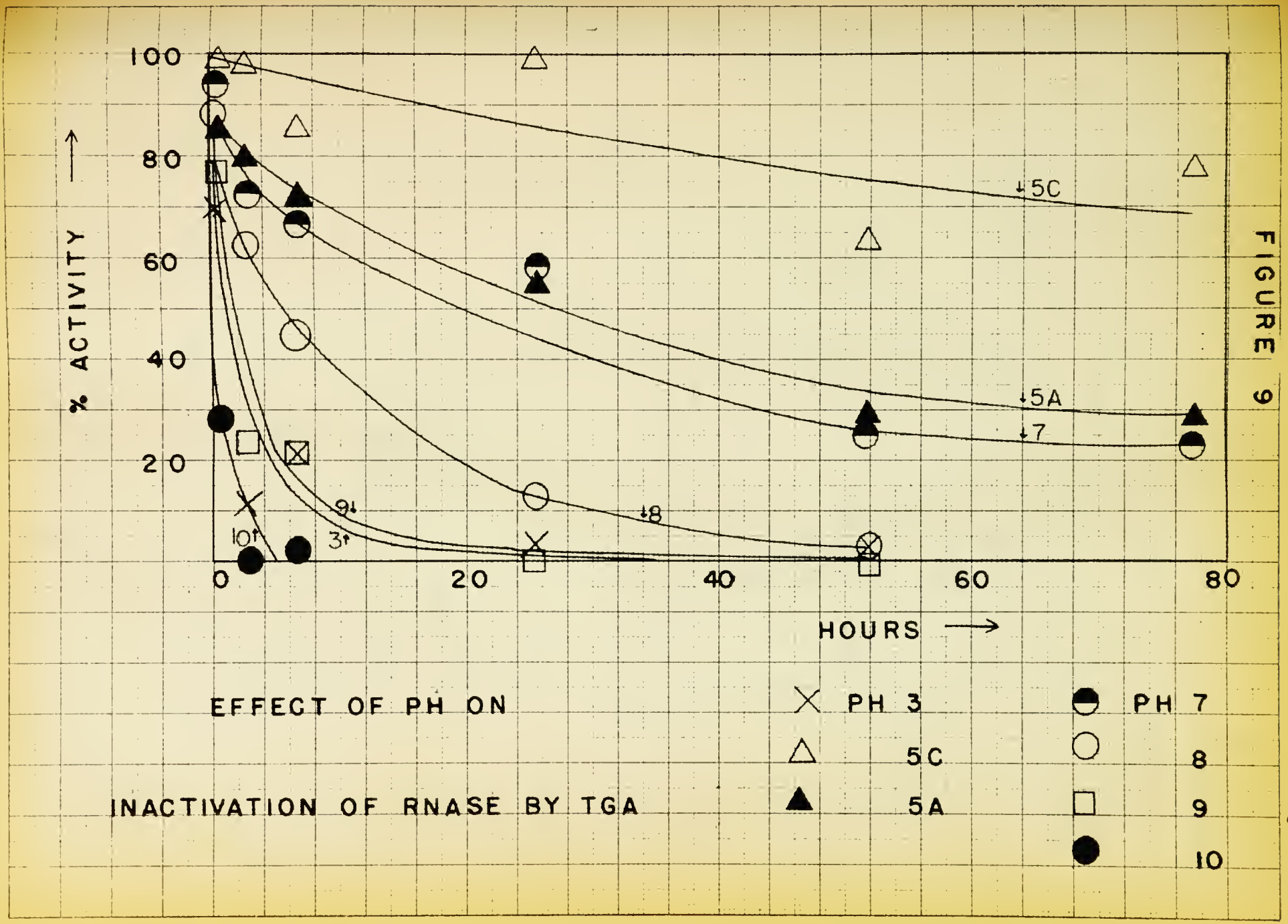
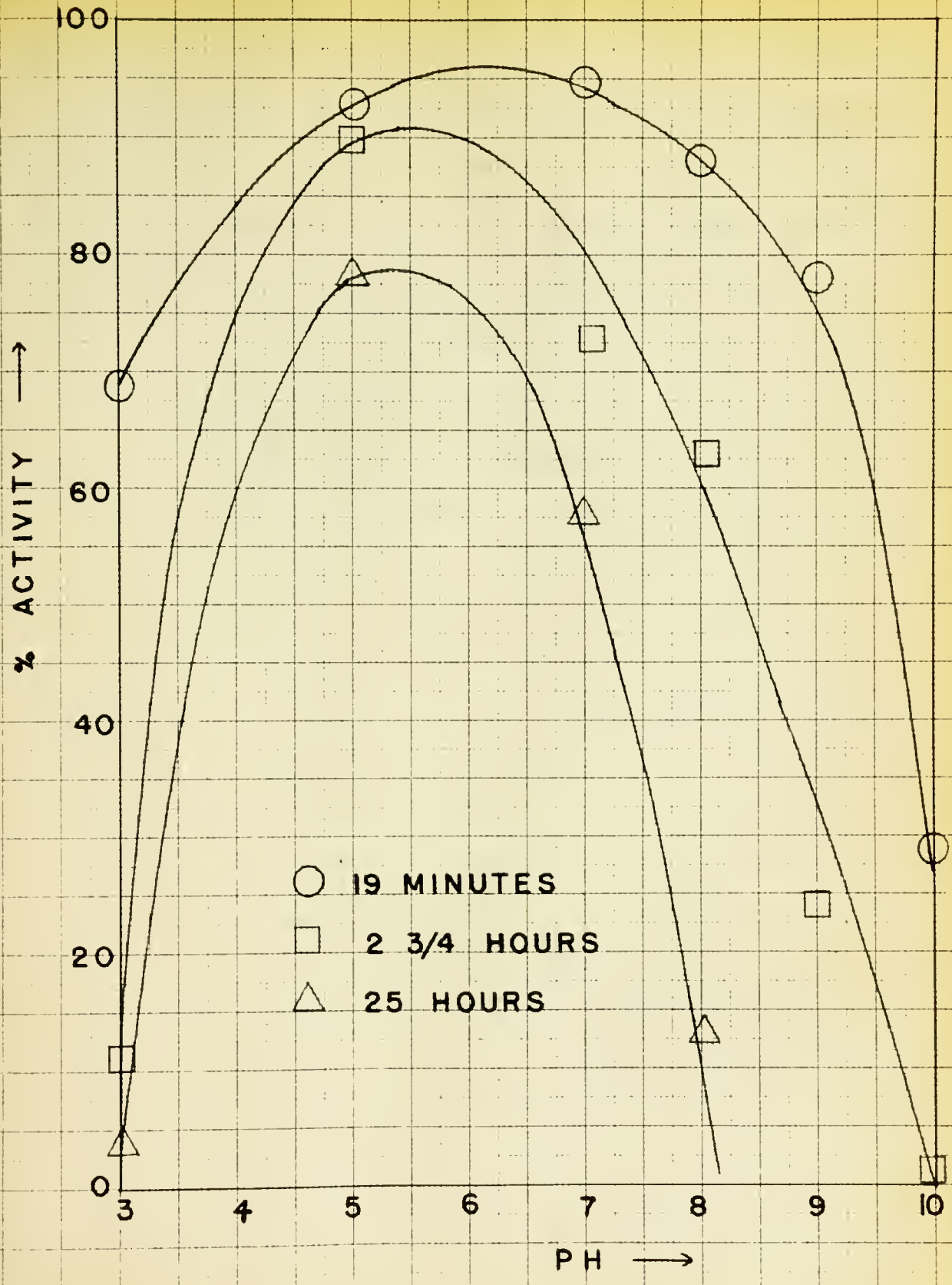

100
80
60
40
20
0
20
40
60
80
% ACTIVITY
HOURS
5C
5A
7
8
9
3
10
EFFECT OF PH ON
INACTIVATION OF RNASE BY TGA
PH 3
5C
5A
PH 7
8
9
10

FIGURE 9

FIGURE 10



EFFECT OF PH ON INACTIVATION OF RNASE BY TGA

employed except at pH 5. Loss of activity occurred more rapidly in the presence of acetate than in the presence of citrate buffer at this pH. Nevertheless, even with acetate buffer, the rate of inactivation at pH 5 was slower than that at any other pH tested. Citrate buffer was also used at pH 3, but activity was lost rapidly at this pH. Control solutions, identical in all respects to the experimental solutions except for the absence of thioglycolate, lost little or no activity during a 55 hour period.

In another experiment, loss of enzyme activity was investigated as a function of both urea and pH. The combinations of hydrogen ion concentration and urea concentration tested are indicated in Table 4 and Figure 11 in which the results of the experiment are presented. The buffers used were glycine at pH 2, citrate at pH 3, 4, and 5, and carbonate at pH 10, each at a concentration of 0.02 M. The concentration of ribonuclease was 0.09 per cent, that of thioglycolate, 0.07 M. The effect of pH on the inactivation of ribonuclease in 4 M urea was much the same as in the preceding experiment. When the urea concentration was increased to 8 M, however, loss of activity occurred rapidly at all pH values tested, the protein being virtually inactive at the end of 4 hours. Nevertheless, measurements after 10 minutes of reduction showed that considerable activity was retained at pH 3 and pH 5, but that at pH 10 the enzyme was already completely inactive. In the absence of urea, only samples of enzyme at pH 10 had lost a significant amount of activity at the end of 28 hours.

At each combination of pH and urea concentration, a control solution was prepared from which thioglycolate was omitted. Significant loss of activity occurred only in that solution of ribonuclease which was treated

Table 4

THE EFFECT OF pH AND UREA ON THE INACTIVATION OF RIBONUCLEASE BY THIOGLYCOLIC ACID

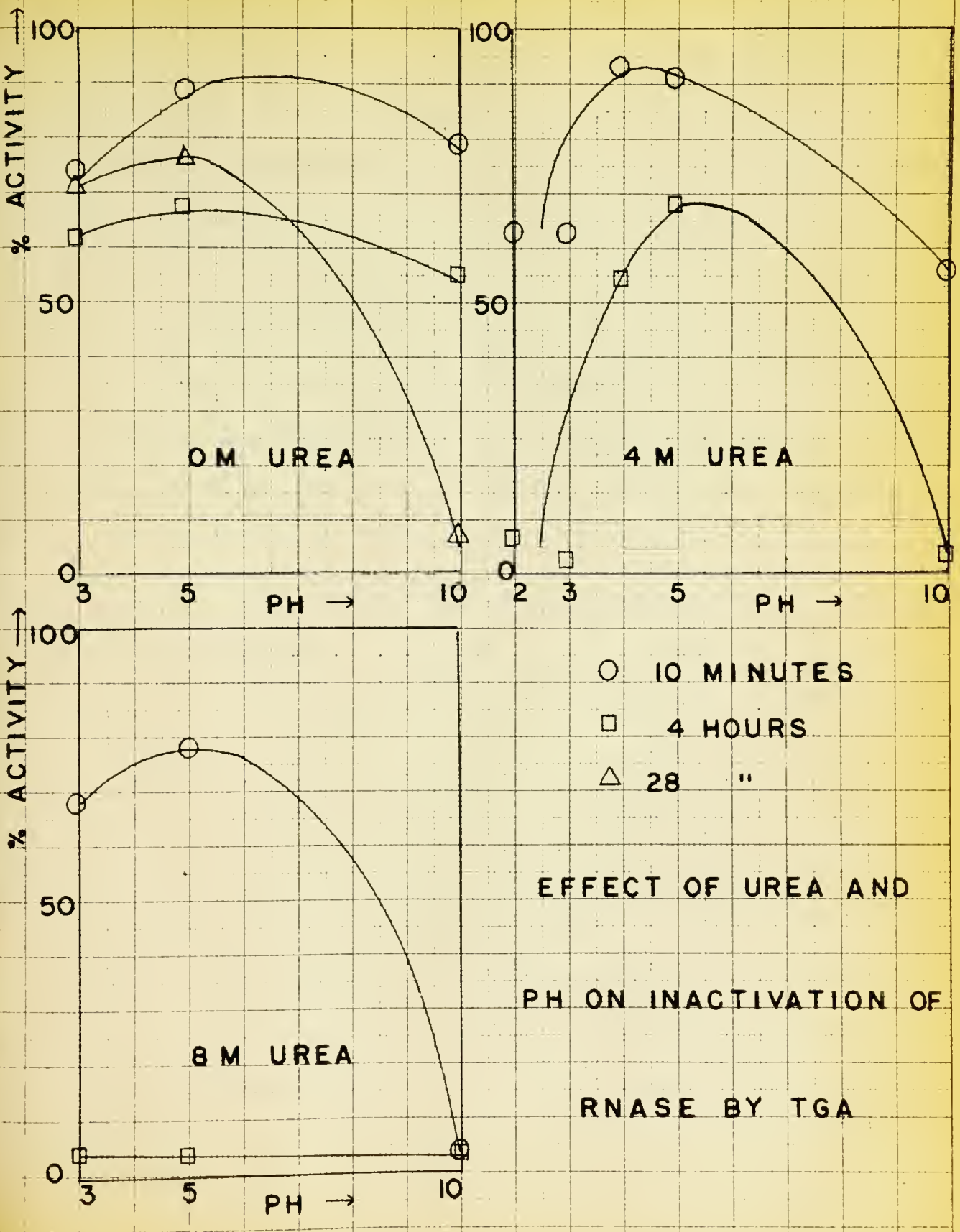
UREA M	pH	PER CENT ACTIVITY				
		10 min.	4 hrs.	14 hrs.	28 hrs.	77 hrs.
8	3	68	4	1	-	-
8	5	78	3	0	-	-
8	10	3	3	0	-	-
4	2	63	6	0	-	-
4	3	62	2	0	-	-
4	4	93	54	33	19	0
4	5	91	68	80	65	72
4	10	56	2	0	-	-
0	3	73	62	74	71	85
0	5	88	67	75	77	91
0	10	79	55	28	8	-

Figure 11

The effect of pH and urea concentration on the inactivation of ribonuclease (RNase) by thioglycolic acid (TGA). There are separate graphs for 0, 4, and 8 M urea. In each graph, per cent activity is plotted against pH with time as parameter.

The conditions of the experiment were: 0.09 per cent ribonuclease, 0.07 M thioglycolic acid, and 0.02 M buffer. Urea concentration and pH are listed in Table 4.

FIGURE 11



EFFECT OF UREA AND PH ON INACTIVATION OF RNASE BY TGA

with 8 M urea at pH 10. The enzyme activity of this solution was 29 per cent at the end of 24 hours, and 8 per cent after 47 hours. Nevertheless, the difference between the activity of this solution, and that of the corresponding sample containing thioglycolate at this pH and urea concentration, was highly significant.

c. Thioglycolic Acid Concentration. Amounts of thioglycolic acid giving final concentrations from 0.09 M to 0.00009 M were added to 0.03 per cent solutions of ribonuclease in 0.02 M tris buffer at pH 8 and 8 M in urea. The 0.09 M solution of thioglycolate contained a molar excess of reducing agent over protein of 4200. As shown in Figure 12, inactivation occurred rapidly at thioglycolate concentrations of 0.09 M and 0.009 M. However, little activity was lost in the more dilute solutions.

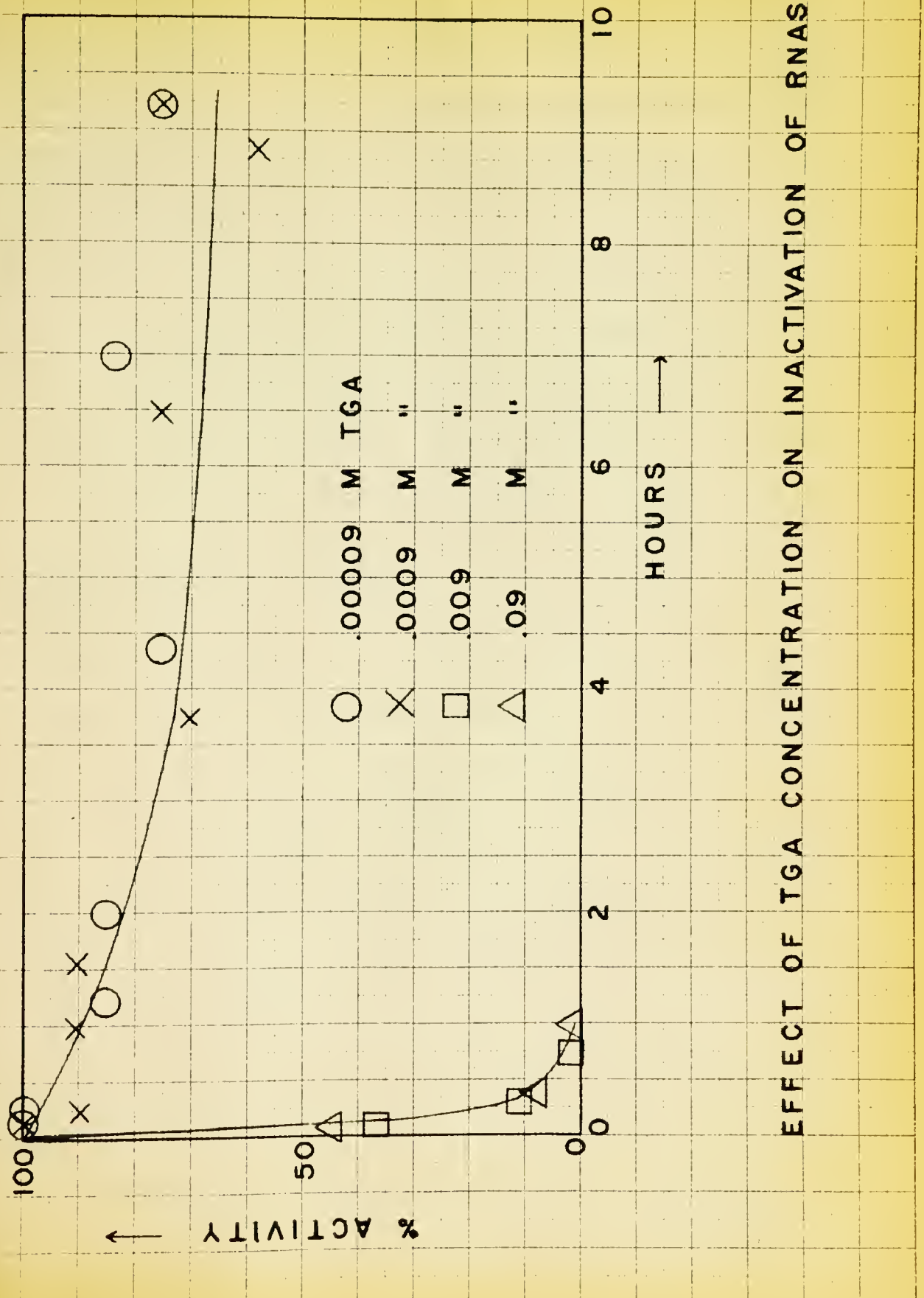
That inactivation of ribonuclease can occur when small amounts of protein are treated with very dilute solutions of thioglycolic acid in the presence of 8 M urea was demonstrated by the following experiment. A 1.0 per cent solution of ribonuclease in 0.05 M tris buffer at pH 8.5 was made 0.3 M in thioglycolic acid and 1.0×10^{-4} M in EDTA No urea was present in the solution. After 9 hours, an aliquot of this solution was diluted 20 fold and a 10 microliter sample of the diluted solution added to 1 ml. of Kunitz substrate which had been made 8 M in urea. This substrate, which, except for the presence of urea was identical to the ordinary Kunitz substrate, will be referred to as "Kunitz urea substrate." (See Section II-C-2,) The optical density of the resulting solution was followed with respect to time. The measured enzyme activity of the sample was 10 per cent of that of an equal amount of native enzyme (5 micrograms of ribonuclease). However, when the assay was carried out in an identical manner using ordinary Kunitz substrate without urea, the

Figure 12

The effect of variation in thioglycolic acid (TGA) concentration on the rate of inactivation of ribonuclease (RNase). Per cent activity is plotted against time with thioglycolate concentration as parameter.

A 0.03 per cent solution of ribonuclease was made 0.02 M in tris buffer at pH 8 and 8 M in urea. The thioglycolate concentrations were 0.00009 M, 0.0009 M, 0.009 M, and 0.09 M.

FIGURE 12


EFFECT OF TGA CONCENTRATION ON INACTIVATION OF RNASE
% ACTIVITY
100
50
0
0
2
4
6
8
10
HOURS
.00009 M TGA
.0009 M "
.009 M "
.09 M "

measured activity of the 10 microliter sample was found to be 80 per cent of that of an equal amount of native enzyme. In contrast, if the protein in a similar preparation of ribonuclease and thioglycolic acid were separated from the reducing agent prior to measurements of activity, samples assayed in the Kunitz urea substrate had about the same enzymatic activity relative to standards of native ribonuclease, as samples assayed in Kunitz substrate without urea.

These two observations indicated that the protein sample was less active when assayed in Kunitz substrate containing both thioglycolic acid and 8 M urea, than when assayed in Kunitz substrate which did not contain either the thioglycolate or the urea. Indeed, it seemed likely that the enzyme was actually partially inactivated during the course of the five minute Kunitz assay. This conclusion was supported by an experiment in which separate aliquots of thioglycolic acid and native ribonuclease were added first to Kunitz urea substrate, and then in equal amounts to Kunitz substrate without urea. The optical density of these solutions was followed with respect to time in the usual manner. It was found that the activity of the enzyme, as measured in the urea substrate, was equal to approximately one third of the enzyme activity as measured in the ordinary Kunitz substrate. Since equal amounts of native ribonuclease had been added to the substrate in each case, the enzyme in the urea substrate must have been partially inactivated during the course of the assay.

The actual concentrations of reagents in the Kunitz urea substrate were 5.0×10^{-4} per cent ribonuclease, 1.5×10^{-4} M thioglycolic acid, 8 M urea, and 0.05 M acetate buffer at pH 5. The results obtained may be compared with those reported by Liener (58) who found that a 0.2 per cent

solution of trypsin lost considerable activity when treated with 1.3×10^{-4} M mercaptoethanol at pH 7.6 in the presence of 8 M urea.

One practical consequence of the experiments on the effect of thioglycolate concentration on inactivation was the demonstration that relatively small variations in sulfhydryl content of the reducing agent had no effect on the rate of reduction, provided that there was a sufficiently large molar excess of thioglycolic acid over protein. It was observed that at alkaline pH the thioglycolate sulfhydryl groups were quite labile, the concentration of measured thiol decreasing by one half over a 24 hour period. However, if the initial concentration of thioglycolic acid were sufficiently high, even such a marked decline in sulfhydryl concentration could safely be ignored.

2. Demonstration of Sulfhydryl Groups

It is expected on theoretical grounds that the inactivation of ribonuclease brought about by treatment with thioglycolic acid will be accompanied by reduction of the cystine residues of the protein. If indeed this is the case, then it should be possible to show that factors such as pH and urea concentration, which control the rate of inactivation, also influence the extent of reduction of the enzyme.

In this paper, per cent reduction always refers to average reduction. Although extent of reduction need not be uniform for all molecules, we were able to measure only the overall sulfhydryl concentration of a sample. For instance, the assay techniques used did not distinguish between a sample of protein half the molecules of which were completely reduced and the other half not reduced, and a sample in which all the molecules were 50 per cent reduced. One hundred per cent reduction means cleavage of the disulfide bonds of all four cystine residues with the

production of eight sulfhydryl groups per molecule of protein.

Measurements of the sulfhydryl content of samples of ribonuclease which had been treated with thioglycolic acid were made subsequent to a procedure (usually ion exchange, rarely alcohol precipitation) which separated the protein from the mercaptan reducing agent. Samples of native protein run through these procedures emerged 100 per cent active and with no sulfhydryl groups measurable by the NEMI assay.

a. Urea Concentration. The effect of urea concentration on the reduction of ribonuclease disulfide bonds is illustrated in Figure 13. The experiments summarized in this figure were usually done on different days but under similar conditions. Solutions of 1.0 to 2.5 per cent ribonuclease in dilute tris buffer at pH 8.0 to 8.5 (reduction in one 8 M urea sample took place at pH 9) were treated with thioglycolic acid in concentrations ranging from 0.2 to 0.35 M. The concentrations of urea in these solutions were 0, 4, and 8 M. The protein was separated from the reducing agent by passage through ion exchange resin (chloride cycle).

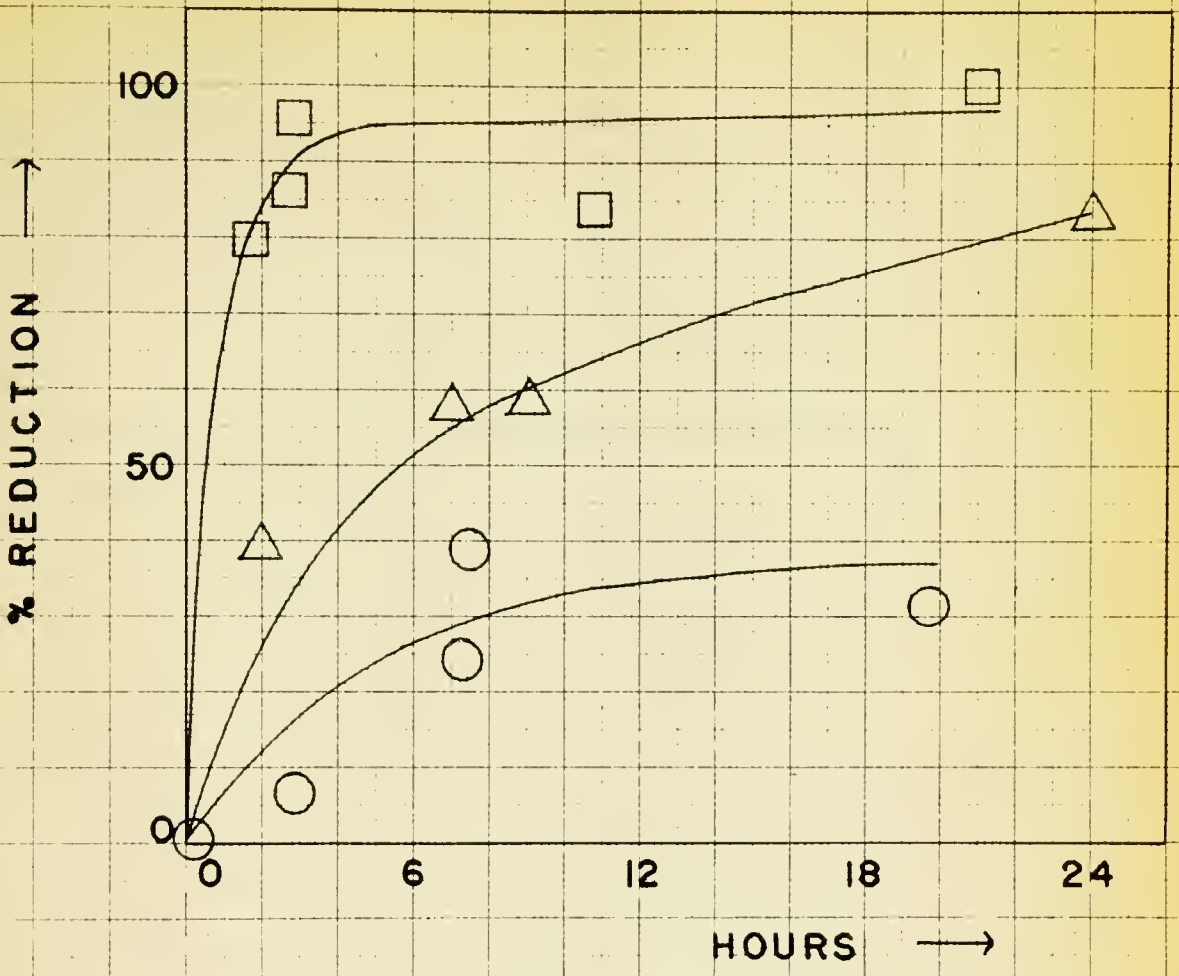
Under these conditions, reduction took place slowly in the absence of urea. After 20 hours, the sample in 0 M urea was 30 per cent reduced. This represented the cleavage, on the average, of a little more than one disulfide bond per molecule. In 4 M urea reduction occurred more rapidly, with about 50 per cent of the disulfide bonds reduced at the end of 6 hours, and 80 per cent at the end of 24 hours. When the concentration of urea was raised to 8 M, 80 per cent of the bonds were broken in 90 minutes. The maximum amount of reduction achieved in 8 M urea was between 85 and 100 per cent. This meant that three or four of the disulfide bonds could be broken under these conditions.

Figure 13

The reduction of ribonuclease (RNase) by thioglycolic acid (TGA) in various concentrations of urea. Per cent reduction is plotted against time with urea concentration as parameter.

The conditions of the experiment were: 1.0 to 2.5 per cent ribonuclease, 0.2 to 0.35 M thioglycolic acid, 0.04 M to 0.05 M tris buffer, and pH 8 to 8.5 (the reduction in one 8 M urea solution proceeded at pH 9). The concentrations of urea were 0, 4, and 8 M.

FIGURE 13



EFFECT OF UREA CONCENTRATION

ON REDUCTION OF RNASE BY TGA

b. Hydrogen Ion Concentration. It has been suggested that the form of the mercaptan thioglycolic acid which is active in reduction is the mercaptide ion (54). Since almost none of the thioglycolic acid is in this form at pH 3, we were led to question the evidence presented in Section III-B-1-b that inactivation of ribonuclease occurred rapidly and completely in such acid solutions. The possibility was considered that the inactivation may have taken place not as a result of reduction, but secondary to some other structural modification of the protein which may have occurred at acid pH in the presence of mercaptan. In an attempt to clarify this issue, the following experiment was performed.

A 2.0 per cent solution of ribonuclease in 0.04 M citrate buffer at pH 3 was made 4 M in urea and 0.3 M in thioglycolic acid. At the time intervals indicated, aliquot volumes were passed through the ion exchange resin (acetate cycle). After the sample had run into the resin, the column was washed with 8 M urea and the effluent samples collected. Initially, it was thought that the reduction reaction would stop as soon as the sample which was added to the column had run into the resin, and consequently that washing with 8 M urea would have no effect on the protein. However, measurements of enzyme activity of the sample before and after passage through the column indicated that further inactivation of the protein had taken place while the sample was on the column in contact with 8 M urea. (In other experiments, in which the concentration of urea used for washing the column was the same as that in the reduction solution, no change in activity occurred as a result of passage through the column.)

Assays for enzyme activity and sulfhydryl concentration indicated that a sample which was added to the column at the end of 18 minutes emerged from the column with 7 per cent of the protein disulfide bonds

reduced and an activity loss of 78 per cent. After 40 hours, 51 per cent of the disulfide bonds had been reduced and the enzyme was completely inactive. Thus, even though the reduction proceeded more slowly than at pH 8 or 9, some reduction of the protein did occur at pH 3.

3. Change in Chromatographic Behavior

Native and thioglycolate-treated ribonuclease were chromatographed on a 0.9 by 30 cm. column with Amberlite resin XE-64 in 0.2 M phosphate buffer at pH 6.35 and 2°C.

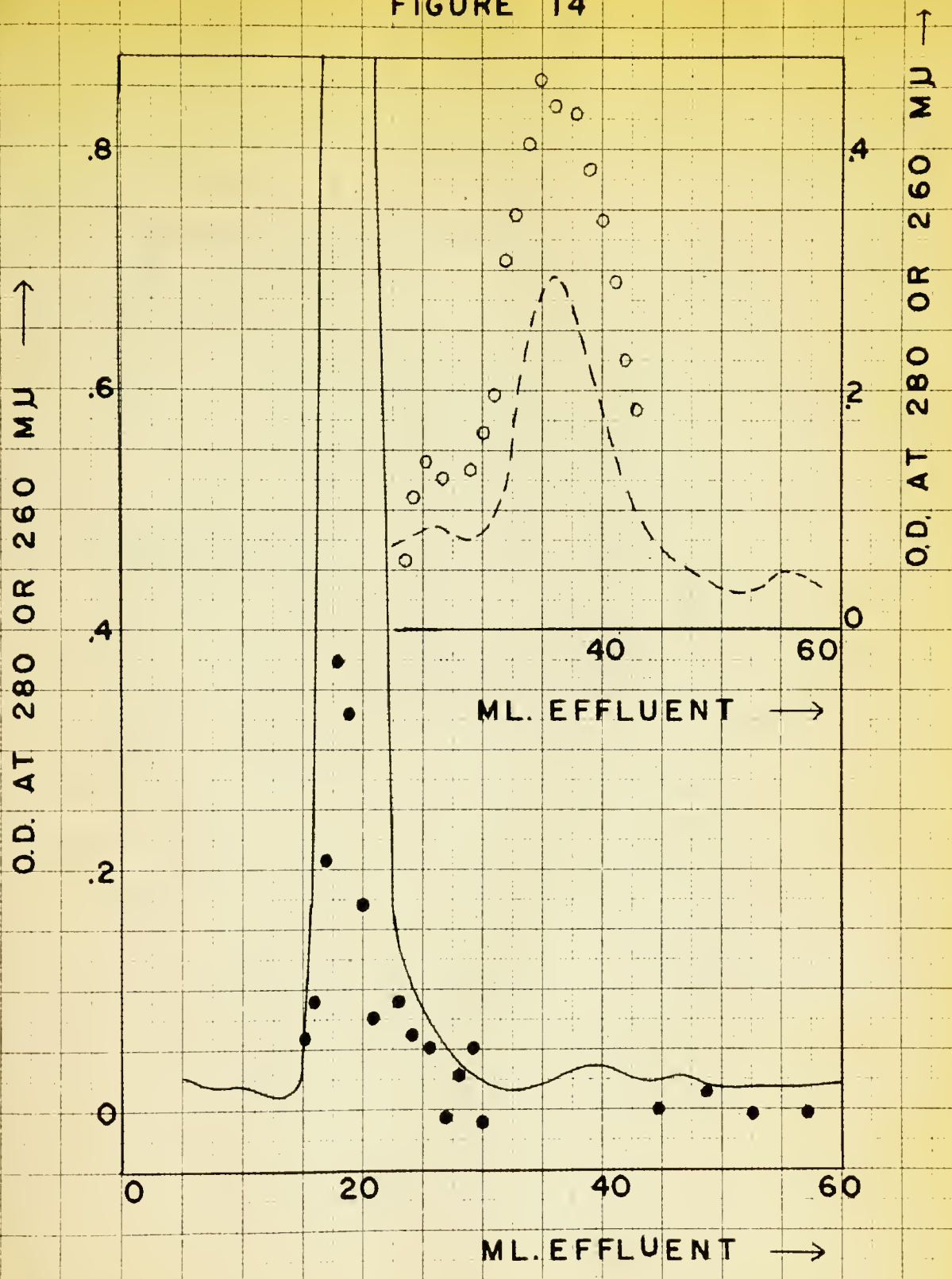
After 30 hours of standing at room temperature, a solution of 1.0 per cent ribonuclease in 0.05 M tris buffer at pH 8 and 0.1 M in thioglycolic acid was made 0.32 M in iodoacetic acid (recrystallized twice from carbon tetrachloride), and the hydrogen ion concentration again adjusted to pH 8. The alkylation was permitted to proceed for 18 hours. Then a 1 ml. aliquot volume was added to the column. The effluent fractions were analyzed for protein by the ninhydrin method (66), and for thioglycolate, iodoacetate, and protein by determination of the optical density at 280 millimicrons. (At 280 millimicrons, a 0.01 M solution of sodium thioglycolate at pH 8.0 has an optical density of 0.23 and a 0.001 M solution of sodium iodoacetate at pH 8.6 an optical density of 0.30.) As indicated in Figure 14, all material having significant absorption at 280 millimicrons was eluted at the column front. Native ribonuclease, when chromatographed in the same system, appeared in a peak between the thirtieth and forty-fifth milliliters of effluent. None of the thioglycolate-treated protein appeared in the position ordinarily occupied by the native enzyme.

Figure 14

A 1.0 per cent solution of ribonuclease (RNase) at pH 8 was treated with 0.1 M thioglycolate (TGA). After 30 hours, the solution was made 0.32 M in iodocetate. The resulting solution was chromatographed on a 0.9 by 30 cm. column with Amberlite resin XE-64, in 0.02 M phosphate buffer at pH 6.35 and 2°C. The optical density at 280 millimicrons of the effluent fractions was determined (solid line). The ribonuclease activity of the effluent fractions was also measured by the Anfinsen assay and is expressed as optical density at 260 millimicrons, corrected for the assay blank (solid circles).

Native ribonuclease was chromatographed in the same system and the resulting effluent fractions analyzed for absorbance at 280 millimicrons (dashed line), and enzyme activity expressed as optical density at 260 millimicrons (open circles).

FIGURE 14



CHROMATOGRAPHY OF
NATIVE AND TGA-TREATED RNASE

The results of other experiments (see Section III-B-2-a) indicate that an average of one or two disulfide linkages may be broken when ribonuclease is treated with thioglycolic acid at pH 8 in the absence of urea. Whether or not the cleavage of these bonds is alone responsible for the dramatic change in chromatographic pattern observed cannot be determined without further study. It is possible that other alterations in secondary structure of the protein may come about as a result of treatment with thioglycolic acid and subsequent alkylation, and that these contribute to the change in chromatographic behavior.

4. Change in Solubility

Although the solubility of reduced ribonuclease was never determined quantitatively, it was observed to be considerably less than that of the native enzyme. The reduced protein was, however, quite soluble in concentrated solutions of urea. For example, a sample of 3 mg. of protein which had been completely inactivated by thioglycolic acid at pH 9 in 8 M urea was not soluble in 1 ml. of distilled water, but went into solution easily in 1 ml. of 8 M urea.

C. Reactivation of Reduced Ribonuclease

Since sulfhydryl groups are quite susceptible to air oxidation, forming the corresponding disulfides, it is tempting to speculate whether oxidation of the sulfhydryl groups of reduced ribonuclease would lead to recovery of some of the enzymatic activity which had been lost on reduction. Several experiments exploring the possibility of such reactivation were carried out. These were in general of two kinds. In one type, a 1 to 2 per cent solution of ribonuclease in 0.04 M tris buffer at pH 8 to 9 was inactivated in the presence of 0.2 to 0.3 M

thioglycolic acid and 2 to 8 M urea. Aliquots of this solution were diluted ten to forty fold and recovery of activity with respect to time was measured. The second type of experiment consisted of the inactivation of the protein under the same conditions, but followed by passage of the solution through ion exchange resin with separation of the reducing agent from the protein. Reactivation of a diluted sample of thioglycolate-free ribonuclease was then studied. Reoxidation was permitted to occur by allowing samples of reduced protein to stand for a time at room temperature.

Although it was demonstrated that a sample of reduced protein did lose some sulfhydryl groups upon standing, in most of these experiments only changes in enzymatic activity were measured. No attempt was made to correlate decrease in sulfhydryl concentration with increase in activity.

Of the two types of experiments described above, reactivation was achieved more reliably when the protein was not separated from the mercaptan reducing agent prior to reoxidation. When the solution of protein and reducing agent no longer demonstrated ribonuclease activity, an aliquot of the solution was diluted as described and its enzyme activity found to increase from 0 per cent immediately after dilution, to a maximum of 30 to 40 per cent at the end of 24 hours. If the protein was diluted after it had lost only part of its activity as a result of treatment with thioglycolic acid, recovery of some activity could sometimes be demonstrated. A typical experiment demonstrating reactivation of reduced enzyme is described below.

A solution of 2.0 per cent ribonuclease in 0.04 M tris buffer at pH 9 was treated with 0.2 M thioglycolic acid and 2 M urea. At various intervals, aliquot volumes were removed and diluted forty fold. The per

cent activity of the enzyme in each of these diluted solutions was then measured both immediately after diluting and after 4 hours, 24 hours, or 4 days of standing at room temperature.

Table 5

REACTIVATION OF REDUCED RIBONUCLEASE

Per Cent Activity of Diluted Solutions			
Immediately after Diluting	4 hours after Diluting	24 hours after Diluting	4 days after Diluting
100		74	
100			70
60		66	
48		64	
48			70
28			70
26			64
8	48		
0			40

As indicated in Table 5, the diluted enzyme samples which were less than 50 per cent active after treatment with thioglycolic acid gained activity upon standing, while those which were initially fully active lost activity on standing. The activity of one sample was 60 per cent immediately after dilution and this was scarcely changed after one day of reoxidation.

In this group of experiments, reduction of the protein took place in solutions containing 1 to 2 per cent ribonuclease, 0.2 to 0.3 M thioglycolic acid, and 2 to 8 M urea. Reactivation of the partially or

completely inactivated enzyme did not occur unless these solutions were diluted so that the final protein concentration was approximately 0.05 per cent with corresponding dilution of the reducing agent and urea. The enzyme in undiluted solutions did not regain any activity on standing. Since dilution of the original solution resulted in a decrease in ribonuclease, thioglycolate, and urea concentrations, it could not be determined whether the dilution of one, two, or all three of these agents was essential in making reactivation possible. To clarify this point, further experiments would have to be done in which the concentrations of these components were varied independently.

Results of experiments in which reactivation was studied after the thioglycolic acid had been separated from the ribonuclease by ion exchange were erratic. A sample of protein which was completely inactive after elution from the ion exchange column was diluted and regained 10 per cent of its theoretical activity in 8 hours. Similar solutions gained almost no activity during this period. In other experiments, thioglycolate-free protein, initially inactive, became 30 to 50 per cent active approximately 24 hours after dilution. Occasionally, samples of protein which had regained some activity, lost it again with further standing at room temperature. In any case, no activity was recovered by samples of protein having concentrations as great as 0.3 per cent, but a five fold dilution of such a solution might regain some activity after several hours.

In summary, reduced ribonuclease, when allowed to stand at room temperature for prolonged periods of time, may regain some of the enzyme activity which was lost as a result of reduction of the disulfide bonds. However, the exact conditions governing this reactivation remain to be defined.

The problem of reoxidation of reduced ribonuclease has also been studied by Anfinsen and collaborators (4, 87). These workers found that completely reduced and fully inactive samples of protein, in concentrations of 0.1 per cent, regained 12 to 19 per cent of their enzyme activities in 68 hours with some decrease in the number of sulfhydryl groups per molecule. In contrast, similar treatment of protein which had been 50 per cent reduced led neither to gain of activity nor loss of sulfhydryl groups.

D. Reactions with Sulfhydryl Reagents

Studies of the structure of reduced ribonuclease by amino acid analysis require that the labile sulfhydryl groups be converted to stable derivatives prior to hydrolysis of the protein. The ideal reagent for such a purpose is one which is specific for sulfhydryl groups, reacting with no other groups of the protein. To test for non-specific reactions, experiments were done in which agents known to react with sulfhydryl groups (sulfhydryl reagents) were mixed with samples of native ribonuclease and the effect on enzymatic activity measured. The agents which were tested were iodoacetic acid, iodoacetamide, n-ethylmaleimide, and silver nitrate.

A solution of 0.05 per cent ribonuclease was treated with 0.1 M iodoacetate at pH 8.0 and the activity followed for 22 hours. In another experiment, ribonuclease was treated with iodoacetamide instead of iodoacetate. As indicated in Figure 15, considerable activity was lost in each case during a 22 hour period, the rate of loss being more rapid for those samples of protein which had been treated with iodoacetamide than for those which had been treated with iodoacetate.

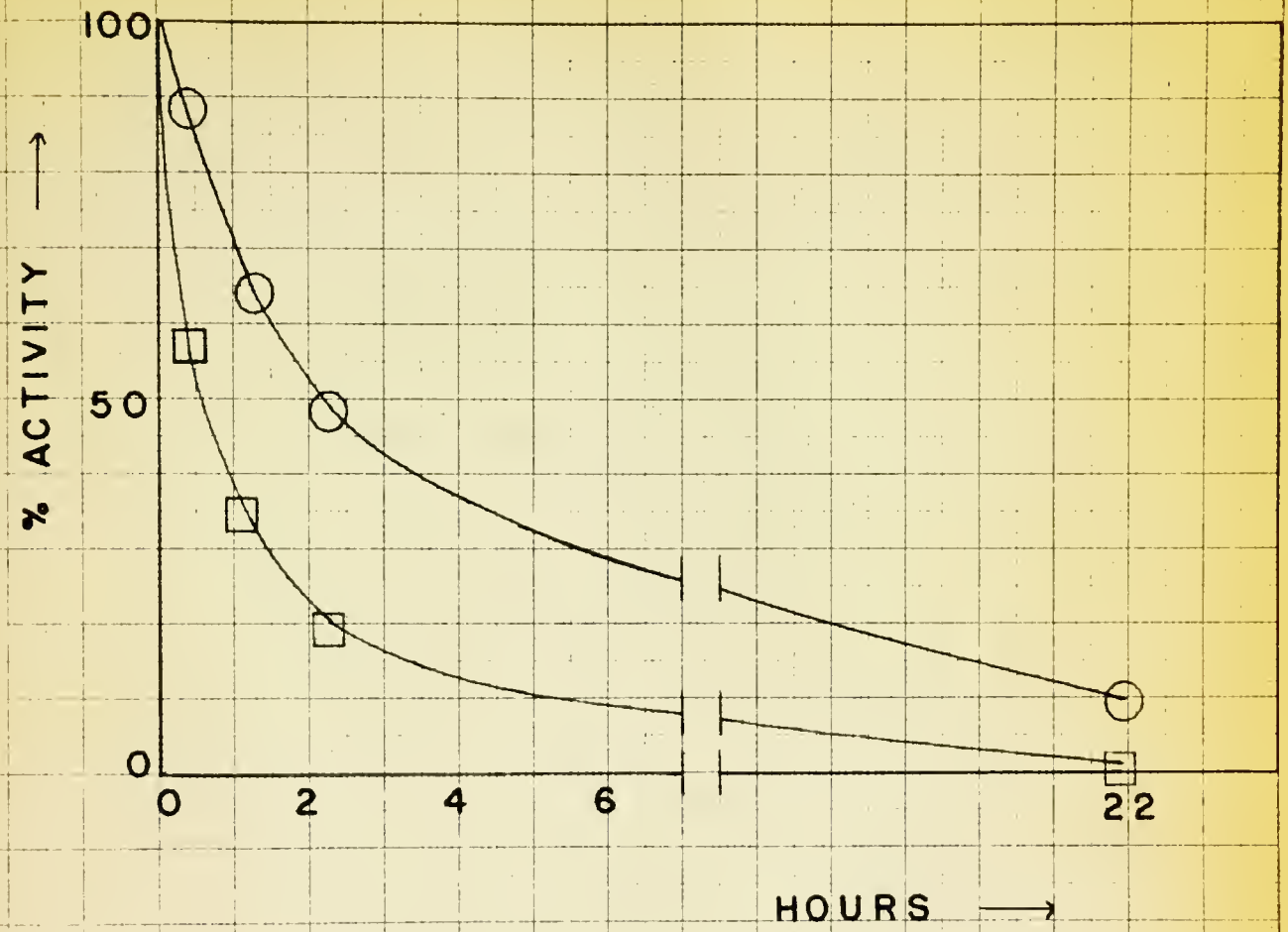
It is well established that native ribonuclease has no sulfhydryl groups. The loss of enzyme activity, therefore, cannot be explained by

Figure 15

The effect of iodoacetic acid (IAc) and iodoacetamide (IAc-NH_2) on the activity of ribonuclease (RNase). Per cent activity is plotted against time.

A solution of 0.05 per cent ribonuclease was made 0.1 M in iodoacetic acid and adjusted to pH 8.0. A similar solution was made 0.1 M in iodoacetamide at pH 8.0. The enzymatic activity of each solution was followed for 22 hours.

FIGURE 15



EFFECT OF IAC AND IAC-NH_2

ON ACTIVITY OF RNASE

the reaction of these agents with sulfhydryl groups on the protein. Furthermore, the reaction with sulfhydryl groups would be expected to occur much more rapidly than the relatively slow inactivation which was observed. However, it has been shown that α-haloacetic acids also react with the hydroxyl group of tyrosine, the ϵ-amino group of lysine, and the imidazole group of histidine forming the carboxymethyl derivatives of these amino acid residues (51), and it may be that reactions such as these were responsible for the losses in enzyme activity which were found. Zittle (104) observed that native ribonuclease was slowly inhibited by iodoacetate and iodoacetamide, but contrary to the experience here, he found iodoacetate to be more inhibitory than iodoacetamide.

These experiments were repeated using silver nitrate and n-ethylmaleimide. It was found that ribonuclease was virtually inactive at the end of one hour when treated with silver nitrate, but that treatment with n-ethylmaleimide for 18 hours did not affect enzyme activity. Of the four agents tested, therefore, n-ethylmaleimide probably has the greatest specificity for sulfhydryl groups.

IV. DISCUSSION

Most of the experimental work in this study has been concerned with the definition of conditions leading to the reduction and inactivation of ribonuclease by the mercaptan, thioglycolic acid. Conditions which were studied were urea concentration, pH, and thioglycolate concentration. It was found that the rate of inactivation of the enzyme, in the presence of mercaptan, increased with increasing urea concentration, particularly when the urea concentration was raised beyond the critical value of 4 M. Rate of activity loss was minimal at pH values near 5, but increased as the pH was changed in either the acid or the alkaline direction. Maximum inactivation of the enzyme was achieved in alkaline solutions of concentrated urea containing an excess of thioglycolate. Lesser degrees of inactivation were obtained in solutions of neutral or slightly acid pH, and in the absence of urea.

The role of the disulfide bond in maintaining the integrity of the ribonuclease molecule was evaluated by studying the enzymatic activity of partially and completely reduced protein. Loss of activity was found to depend not only on the number of disulfide bonds which had been reduced, but on the method by which this reduction was achieved. It appeared that there was no necessary correlation between extent of reduction and inactivation of the enzyme. Evidence in support of this conclusion will be presented in the following paragraphs.

One way of obtaining information on the relationship between inactivation and reduction is to combine the data presented in Figures 7 and 13. The two families of curves in these figures give values for residual enzyme activity and extent of reduction separately as functions of time with urea concentration as parameter. For each concentration of

urea, the per cent activity and per cent reduction after a given interval of time may be read from the appropriate curve and plotted on a single graph. Figure 16 is such a plot of activity against reduction for 0 and 4 M urea. The points expressing per cent activity as a function of reduction for 4 M urea fall along one curve, and the corresponding points for 0 M urea along an adjacent curve.

This method for establishing a correlation between inactivation and reduction, although adequate for a first approximation, has one serious drawback. The information needed to obtain each point on the activity-reduction graph is the sulfhydryl concentration and the enzymic activity of solutions of identical composition with respect to urea concentration, pH, temperature, etc.. If the values for reduction and loss of activity were obtained by assaying two separate solutions, as was the case in the experiments leading to Figures 7 and 13, it is very difficult to be certain that the composition of the two solutions was indeed exactly the same. For example, since the experiments were done at room temperature, the temperature variable was not rigorously controlled. It was mentioned in the description of Figure 13 that the pH at which these experiments were carried out varied from 8.0 to 8.5. In contrast, the pH of the experiments summarized in Figure 7 was always 8.0. Certainly, reduction at the end of two hours in 4 M urea, pH 8.5 cannot be compared meaningfully with loss of activity at the end of two hours in 4 M urea, pH 8.0. It was thought advisable, therefore, to determine the relationship between reduction and inactivation by measuring both the enzyme activity and the sulfhydryl concentration of a single solution. The use of such a solution for both assays eliminates distortions in the activity-reduction relation which may be introduced by the uncontrolled differences which would almost

Figure 16

A plot of per cent activity versus per cent reduction for ribonuclease which was treated with thioglycolic acid at 0 and 4 M urea. The values for activity and reduction at each concentration of urea were obtained by extrapolation from the corresponding curves in Figures 7 and 13.

See text for details.

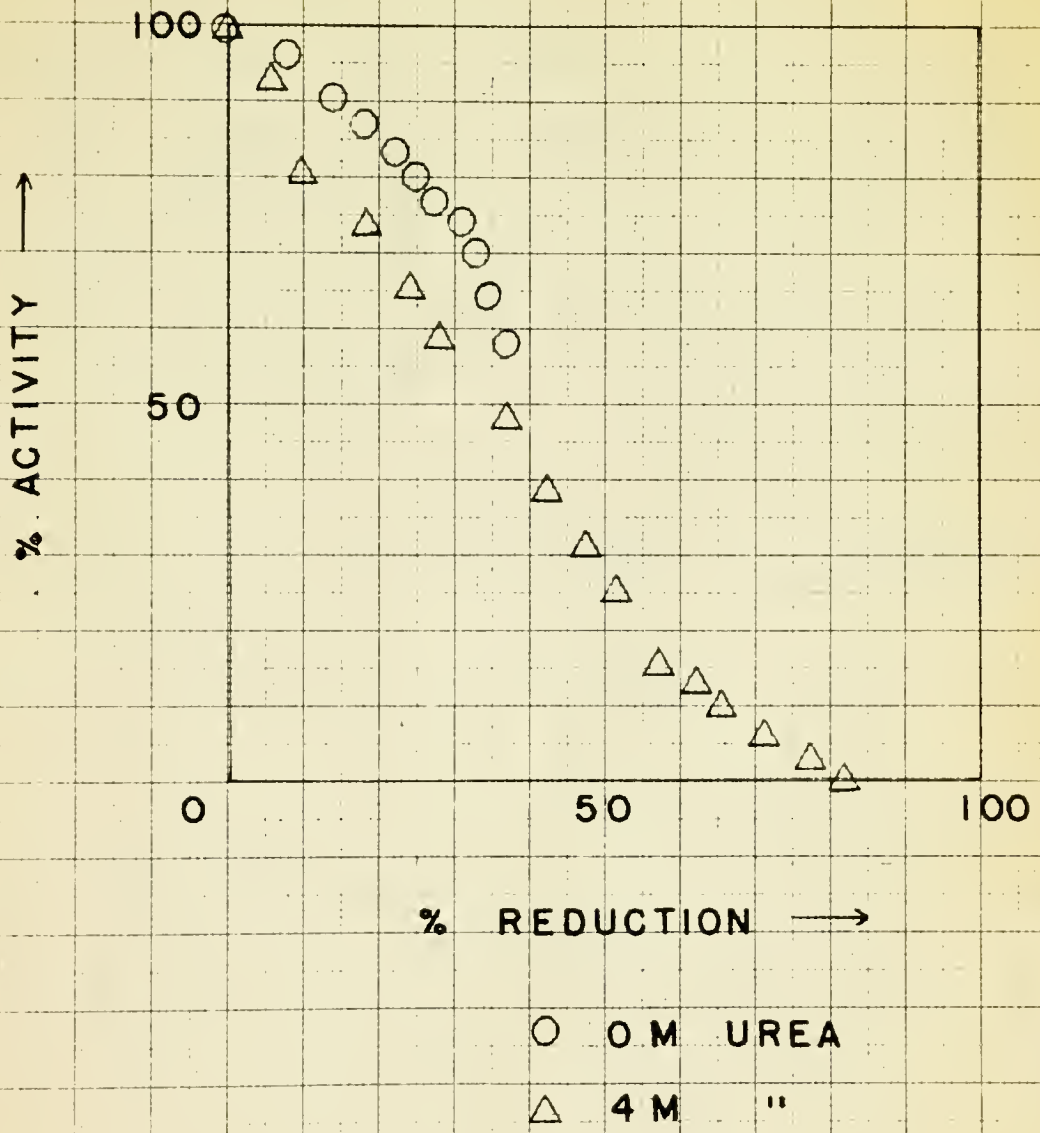
Figure 17

A summary of all experiments in which ribonuclease was treated with thioglycolic acid under a variety of conditions of pH and urea concentration, and the resulting solution assayed both for enzyme activity and sulfhydryl concentration. Per cent activity is plotted against per cent reduction. Each symbol represents a specific set of conditions of ribonuclease concentration, thioglycolate concentration, pH, and urea concentration. Each dashed line is drawn through a set of points represented by a single symbol and gives activity as a function of reduction under one particular set of conditions.

Prior to activity and sulfhydryl assay, the reducing agent was separated from the protein by anion exchange except in the set of experiments indicated by the half-shaded circles in which separation was accomplished by alcohol precipitation of the protein.

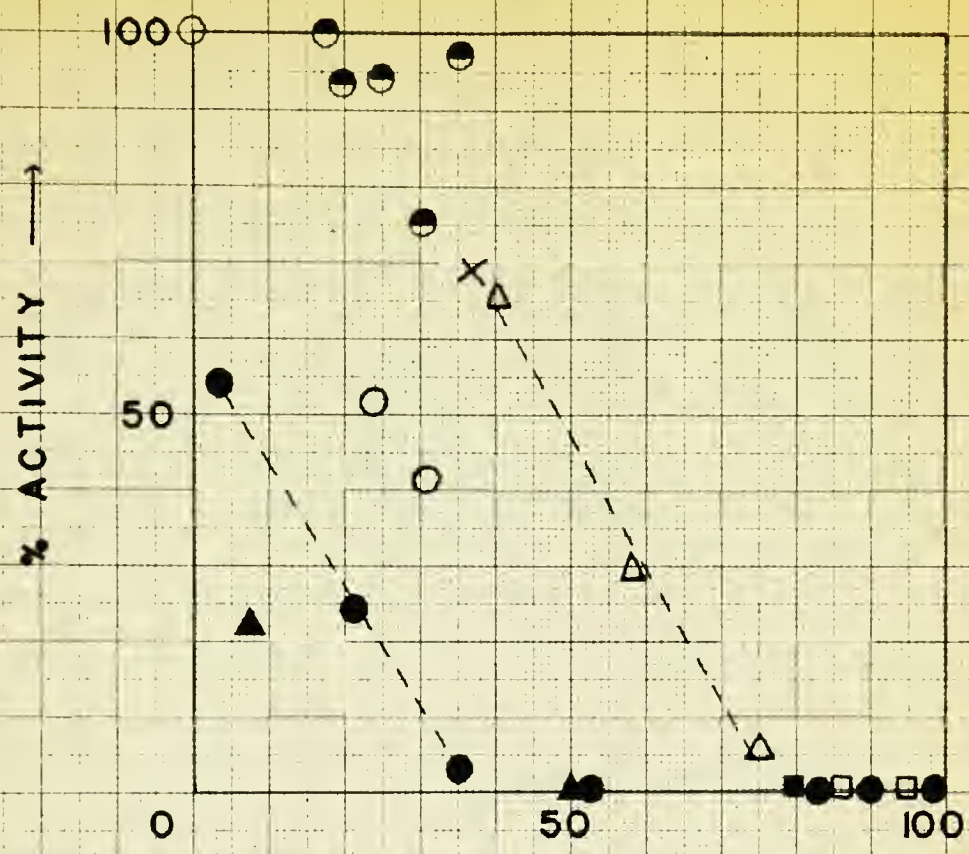
See text for details.

FIGURE 16



ACTIVITY VS. REDUCTION

FIGURE 17



○ 0 M UREA, PH 8
△ 4 M " " 8
× 6 M " " 8
□ 8 M " " 8
● 2-8 M UREA, PH 9
■ 8 M " " 9
▲ 4-8 M " " 3
◓ 0 M " " 8

ACTIVITY VS. REDUCTION

invariably be found between two separate solutions, one of which is assayed for activity and the other for sulfhydryl concentration.

A number of experiments were done under a variety of conditions of pH and urea concentration in which both the enzymic activity and the extent of reduction of a single sample were measured. The points obtained as a result of these experiments are assembled in Figure 17. Each symbol in the figure represents one set of conditions of ribonuclease concentration, urea concentration, pH, and thioglycolate concentration. Usually, a single solution was assayed at various periods of time, thus obtaining the several points depicted by each symbol. Upon examining Figure 17, several facts become apparent.

The points in this figure are widely scattered, implying that in general, there probably is no unique relation between reduction and inactivation. On the other hand, points obtained from an experiment involving a specific set of conditions frequently fall along a straight line. Two such lines are drawn in the figure. Thus, for a given set of conditions, there is a definite, in fact often a linear relationship between inactivation and reduction. However, when the results of experiments in which reduction proceeded under different sets of conditions are compared, this relationship breaks down.

The position of most of the points in Figure 17 indicates that, although there is no unique correlation between inactivation and reduction, it is usually not possible to obtain much loss of activity without some simultaneous reduction. Complete inactivation of the enzyme is usually accompanied by the reduction of at least 80 per cent of the disulfide bonds. However, this generalization does not apply to the 2 groups of points representing reduction at pH 9 in "2-8 M urea" and reduction at pH 3 in "4-8 M urea."

These points summarize the results of several experiments in which ribonuclease was treated with thioglycolic acid in solutions 2 M in urea at pH 9 or 4 M in urea at pH 3. Samples of partially reduced protein were then added to the ion exchange column and the column washed with 8 M urea. The effluent from the column was found to have less enzyme activity than the sample originally added to the column. (See Section III-B-2-b.) As indicated in Figure 17, these effluent samples were less active relative to the extent of reduction than were samples of partially reduced protein obtained in other experiments. It would seem that the protein had been inactivated by some means other than reduction. It is possible, for instance, that in 2 M urea a single protein disulfide bond was reduced. The cleavage of one such bond may not have resulted in extensive changes in secondary structure. However, when the partially reduced protein was exposed to 8 M urea on the column, it may have been denatured irreversibly, thus losing activity without further disruption of disulfide bonds. Indeed, since the thioglycolate was rapidly absorbed by the ion exchange resin, it seems probable that little if any reduction of the protein took place on the column.

The inactivation of the protein which occurred in acid solutions of reducing agent and urea may be understood, perhaps, in similar terms. It has already been mentioned that the reduction of disulfide bonds is expected to take place much more rapidly at alkaline than at acid pH. It was found, however, that the protein, when treated with thioglycolate, lost activity almost as rapidly at pH 3 as at pH 10. Although some reduction did occur at pH 3, the relative loss in activity was much greater than the extent of reduction. The reasons for this inactivation are not understood, but one possibility is that a limited amount of

reduction at this pH was accompanied by denaturation of the protein which was responsible for the marked activity loss. (Although inactivation at pH 3 was demonstrated on many occasions, the only experiment in which both inactivation and reduction at pH 3 were measured was complicated by the change in urea concentration, from 4 M to 8 M, mentioned in the preceeding paragraph. Even though this complication makes interpretation of the experimental results difficult, inspection of Figure 17 reveals that the extent of inactivation, relative to a fixed amount of reduction, was greater at pH 3 in "4-8 M urea" than at pH 9 in "2-8 M urea." Further experiments will have to be done to determine more precisely the extent of reduction at acid pH.)

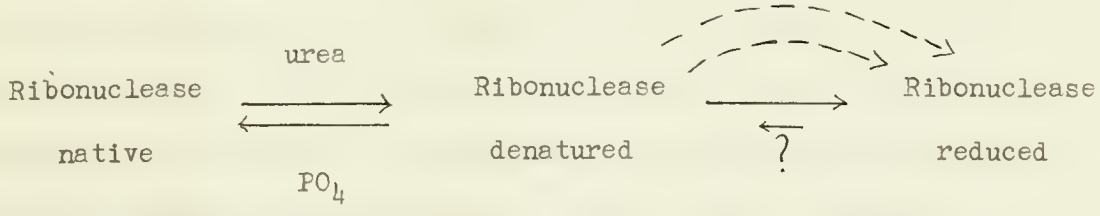
The data presented in Figure 17 may be compared with the results obtained by Anfinsen and collaborators already referred to in Section I-C-3-b. These investigators studied the reduction of ribonuclease by thioglycolic acid at pH 8.5, both in 0 M and in 8 M urea. It was claimed that under these conditions, the reduction of one or two disulfide bonds could be accomplished without significant loss of activity. Other workers (13) have repeated Anfinsen's experiments, but have not been able to confirm his results. In the present study, it has usually not been possible to achieve much reduction without concomitant loss of activity.

It is possible that the differences between Anfinsen's results and our own may be explained by the hypothesis that the properties of the reduced protein, including its biological activity, depend on the path by which reduction is attained. Some methods of reduction, for instance, may be accompanied by more extensive denaturation than others, with consequent differences in the enzymatic activity of the resulting protein. It becomes apparent then, that the results of treatment with agents such as thioglycolic acid must be interpreted in terms of the effects of such

agents not only on the disulfide bonds, but on all aspects of the secondary structure of the protein.

In a simplified fashion, one may conceive of the ribonuclease molecule as a folded polypeptide chain, the arrangement of which is constantly shifting, with weak secondary bonds breaking and reforming. The momentary unfolding of a portion of the molecule near a disulfide bond may enable the reducing agent to penetrate to the disulfide site and to reduce the bond. The ability of urea to break hydrogen bonds shifts the equilibrium in the direction of the unfolded molecule and greatly facilitates reduction. The data obtained on the effect of phosphate in inhibiting the inactivation of the enzyme by thioglycolic acid (see Section III-B-1-a) indicate that phosphate ions probably stabilize the molecule in its folded configuration and thus prevent the reducing agent from reacting with the disulfide bond. The reduction of disulfide bonds usually results in loss of enzymatic activity, the extent of this loss in each case depending in part on the number of bonds broken, and in part on the changes in the secondary structure of the protein which accompany reduction.

The interrelation between the native, the reversibly denatured, and the reduced protein may be summarized as follows: (Based on similar formulations by Liener (58) and Klee (48)).



The dashed arrows represent different possible paths of reduction, leading to proteins which are reduced to the same extent but which differ in other properties. The short arrow with the question mark is inserted

to indicate that reoxidation of the reduced protein may occur, with reappearance of some of the activity lost on reduction. That such reactivation occurs under certain circumstances has been indicated by our work and that of Anfinsen, but the exact conditions remain to be defined.

It would be of considerable interest to study in more detail the different paths leading from native to reduced and inactivated ribonuclease. Of special interest is the mechanism of the inactivation of ribonuclease in acid solution of mercaptan. The nature of the reaction between agents containing disulfide groups and mercaptans in acid solution is not completely understood. It would be important to determine whether disulfide exchange reactions (see Section I-C-3-b) of the sort that occur at alkaline pH, also take place at acid pH. For the case of ribonuclease, this problem could be investigated by attempting to isolate the mixed disulfide of cysteine and thioglycolic acid from ribonuclease which has been treated with thioglycolate. Such a mixed disulfide would be formed during the course of the disulfide exchange reaction. Techniques for identifying this compound have been described (54).

That such exchange reactions probably do not occur at acid pH is indicated by the work of Lamfrom and Nielsen (54). These investigators showed that in the exchange reaction between cystine and thioglycolic acid, the active form of the mercaptan was the mercaptide ion. This anion would not be present in significant amounts in acid solution. In addition, Ryle and Sanger (79) found that the exchange reaction between L-cystine and dinitrophenol labeled L-cystine in acid solution was inhibited by thiols. On the basis of available evidence, therefore, it seems likely that disulfide exchange does not occur in acid solutions of

ribonuclease and thioglycolate. The inactivation, as well as the limited degree of reduction found, probably comes about by means of some other, as yet undefined, mechanism.

The possibility that the protein is denatured in acid solution in the presence of mercaptan has already been mentioned. It would be of interest to examine this possibility and, indeed, to investigate the secondary structure of modified protein obtained by reduction under a variety of conditions. Additional information about the reduction reaction could be obtained by studying the rate and order of cleavage of the individual disulfide bonds of the protein. For instance, it would be important to determine the effect of pH on the relative facility with which these bonds are broken. It seems clear that such detailed studies will be necessary if the complex interrelation between reduction of disulfide bonds and enzyme activity is to be understood.

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